THE INFLUENCE OF ARBUSCULAR MYCORRHIZA ON THE WATER RELATIONS OF TREES

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Abstract

Arbuscular mycorrhiza are associations between soil-inhabiting fungi and plants, which have beneficial effects on aspects of plant health and growth. An area of influence of mycorrhiza is the occasional improvement in host tolerance of drought. However the effect of mycorrhizal colonisation on plant water relations remains inconclusive.

This study increases the body of knowledge on the drought response of plant-fungal associations. Hybrid black poplar *Populus* x *canadensis*, a species commonly seen in shelter belts and small plantations, is tested in its drought response to mycorrhizal colonisation. It also suggests an alternative mechanism for the alteration of plant water relations by arbuscular mycorrhiza. The concept of chemical signalling compounds which control plant response to stress, including drought stress, is topical. This study aims to extend that concept to the plant-fungus symbiosis. The ability of the fungal hyphae to act as an integral part of the root system is tested in its contribution to signalling of drought stress to the host shoot.

The response of mycorrhizal poplar to gradients in water availability was tested in two ways. First the effect of changing water availability over time, as drying and wetting cycles, was examined. Second, gradients in water availability across the root-fungal system were considered. The response of poplar to drought stress was tested when inoculated with one of four species of mycorrhizal fungi. These were *Glomus intraradices, Glomus mosseae, Gigaspora rosea* and *Gigaspora margarita.* There was no consistent improvement in plant response to drying. However there were variations in plant response over time, and with severity of drought conditions, particularly by plants colonised with *G.intraradices* and *Gi.rosea.* Changes in the host plant nutrient status were also found.

The concept of hyphal to plant shoot signalling of drought was tested with poplar inoculated with *G.intraradices*, grown in a specially designed microcosm. Fungal hyphae were able to grow into a separate volume of soil from which plant roots were excluded. The soil water availability around plant roots and adjacent hyphae was altered independently. The plant response in terms of gas exchange was monitored under conditions of varying water availability in each section of the root-hyphal system. In

general there were no consistent alterations in plant gas exchange with changing water availability. However during one experiment possible evidence for short-term hyphal signalling to host plants was noticed.

This method is suggested as a new concept for further experimentation in plant-fungal water relations.

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CHAPTER 1

Introduction

1.1 Water, arbuscular mycorrhizal fungi and trees

Water is one of the most important environmental factors influencing the survival and growth of plants. It is clear that under similar climatic conditions, different plant species respond quite differently in their growth and development. Lack of water severely limits plant growth in temperate as well as tropical regions. Plants have developed a number of mechanisms or attributes to cope with lack of water. Drought resistant characteristics can be found in the shoot, the root system, or the plant as a whole. This report focuses on some of the below ground processes which determine the ability of plants to tolerate drought.

Mycorrhiza is a mutualistic symbiosis between soil-borne fungi and plant roots. A large number of species of fungi can form this non-pathogenic association with plants. There are two main types: ectomycorrhiza and endomycorrhiza, which are grouped according to morphological characteristics. In general ectomycorrhizae form a mantle of fungal tissue around the plant roots, which does not occur in endomycorrhizae. However there are subgroups of endomycorrhizae which do possess this hyphal mantle. In ectomycorrhizae the fungus grows intercellularly in the cortex of the plant roots. In endomycorrhizae the fungus grows both intercellularly and intracellularly and forms distinctive structures within the cortical cells known as arbuscles. Broadly, ectomycorrhizae tend to occur in coniferous and some temperate broad-leaved trees. They are members of the Ascomycetes and Basidiomycetes. Endomycorrhizae are more widely distributed among plant species, and geographically. They form in tropical tree species, temperate trees and shrubs, herbaceous and graminoid plants. The most widely spread type of endomycorrhiza are the arbuscular mycorrhizae (AM). They form a distinct group known as the Endogonaceae. A large number of tree species have been found to form associations with arbuscular mycorrhiza, reviewed by Harley and Harley (1987). There has been

considerable work on the role of these mycorrhiza in plant growth and health. However it is less clear their importance in water uptake.

Trees carry on the same processes in their water relations as other plants, but their larger size, slower maturity, and longer life span accentuate certain features, as compared with smaller plants having a shorter life span. The most obvious difference between trees and herbaceous plants in terms of their water relations, is the greater distance over which water must be translocated. Work must be performed to raise water against the force of gravity. This effect can be ignored in short vegetation but not in trees. It is an additional component when considering the water potential of trees. Because of the increase in wind velocity with height the leaves of tall trees tend to transpire more rapidly than those of shorter plants. The 'sun' leaves at the top of the tree are exposed to greater water and temperature stresses than leaves lower down or shaded; that is the leaves which require the most water are furthest from the point of supply, the soil. Tall trees require a vascular system which is able to deliver water rapidly to those parts of the canopy which are most active in transpiration. A large leaf area is supported and supplied by a single trunk. This means that the water relations of a large number of leaves depend on the normal functioning of the vascular system of a single trunk. These points indicate the usefulness of tree species in studies of water relations in mycorrhiza.

Areas of research to be considered in this report are the requirement for water of different species, and the alteration in water requirement induced by mycorrhizal colonisation. The function of arbuscular mycorrhizal fungi will be considered, in terms of their potential for water uptake. In addition, a number of experimental techniques which are being used in this research will be discussed.

1.2 Measurement of water in plant and soil systems

Water movement in the soil, plant and atmosphere is as a result of its free energy. The free energy of water is expressed in terms of its water potential (ψ). This is based on thermodynamic principles derived in Slatyer (1967) and Meidner and Sheriff (1976). Broadly water potential can be defined as the free energy per unit volume of water, assuming the water potential of pure water to be zero at ambient temperature and atmospheric pressure. Energy per unit volume has the same dimensions as pressure, so that plant and soil water potentials are expressed in pascals (Pa).

1.3 Components of water potential

Water potential is lowered below zero, below that of pure water, by dissolved solutes. This constitutes the osmotic potential.

Water is also lowered below that of pure free-standing water due to its properties of surface tension and cohesion. These two forces which retain water in capillaries, constitute the matric forces.

The osmotic and matric forces are considered to be independent. They are known as osmotic and matric potential and make up part of the total water potential.

Hydrostatic pressure is the outward pressure a liquid exerts when at rest. It is another component of the total water potential. It gives rise to a positive potential and so lessens the degree of negativity of the total water potential. However this effect is of importance on only a limited number of occasions during guttation, so it is not commonly included in calculations of water potential.

The gravitational potential is that exerted on water due to its height above sea-level. It is the energy required to raise the water against the downwards force of gravity, and is of magnitude 0.1 MPa for every 10 m above sea level in height. This is of little consequence in most situations, but is of relevance in studies on trees. Hydrostatic and gravitational potentials can effect the total water potential in plant-soil systems, but osmotic and matric potentials are the most important in plant experimentation.

1.4 Water in soil

The availability of water for uptake by plants is determined by the water-retaining capacity of a soil. Soils consist of mineral particles of varying diameters, classified as clay (0.002 mm), silt (0.002-0.06 mm), and sand (0.06-2 mm). These are bound together into aggregates by organic matter and clay particles. Within and between these aggregates there is a network of interconnected spaces of varying diameter. Water and air occupy these spaces. The volume of water and air varies according to the proportion of particles of different sizes, their packing and the demand for water from plants.

The concentration of solutes in water in the soil is generally low, except in saline soils. Thus the major forces retaining water in soil pores are the matric forces. The matric potential is inversely proportional to the diameter of the soil pores (Russell 1973).

Water is drawn only weakly from large soil pores by gravitational potential. After gravitational water has drained from a water-saturated soil, all the soil pores of less than 60 μm are filled with water retained by matric forces (Russell 1973). In this state the soil is said to be at field capacity. It contains the maximum amount of water that it can hold against gravity. Water potentials of -0.01 to -0.03 MPa are typical in soils at field capacity.

1.5 Measuring soil water characteristics

A moisture characteristic curve for a particular soil is determined by its constituent particles, the soil texture, and their packing, the soil structure. The tendency for a soil to retain or release water is described in terms of its water potential. At high water potentials between 0 and –0.1 MPa, the amount of water retained by a soil depends on capillary effects and is therefore strongly influenced by soil structure. At lower potentials, the effect of structure is much less pronounced (Campbell and Gardner 1971) and the shape of the moisture characteristic curve depends more on the soil texture. When laboratory measurements of matric potential are made, these may be different from a field situation, particularly for wet soils,

because of changes in the soil structure when collecting the soil sample. However disturbance of soils at potentials below –0.1 MPa is likely to have little effect.

A moisture curve is usually obtained by drying. The relation between matric potential and water content is not unique. It varies whether the soil is drying or wetting. This effect is termed hysteresis. It is attributed to nonuniformity of pores, differences in radius of curvature between advancing and receding menisci, the effects of trapped air, and differential changes in soil structure during absorption and desorption of water. The hysteresis effect is more pronounced with fine-textured than coarse-textured soil.

There are a range of methods available for monitoring soil water. The relative merits of these are discussed below. The soil water variables measured are determined by the properties of the instrument used for measurement.

1.5.1 Gravimetric method

The gravimetric method determines the water content (θ) of the soil. The gravimetric water content is obtained from the mass of water lost on drying a soil sample to a constant mass.

The water content is calculated as the fraction of the mass of water in the dry mass of soil. This is given as;

Gravimetric water content $\theta_{mass} = m_{wet}/m_{dry}$

It is usually expressed as a percentage of the dry weight of the soil.

Alternatively the water content can be expressed as a volume of soil.

Volumetric water content $\theta = \theta_{mass} \cdot \rho_{dry}/\rho_{water}$

where ρ_{dry} = dry density of soil

ρ_{water} = density of water = 1 M g m^{-3}

Soil moisture release curves are generally presented using volumetric water content. Gravimetric determination of water content can be converted to volumetric water content using the above equations.

The amount of water lost in drying increases with the drying temperature in soils that contain clay or organic matter, and therefore the temperature should be maintained at constant level of $80^{O}C$.

1.5.2 Tensiometers

Tensiometers measure soil water potential (ψ) directly. They consist of a porous ceramic cup on the end of a tube. The porous cup allows water and solutes to pass between an internal solution and the soil, but prevents the passage of solids and air. The soil solution is allowed to equilibrate with that in the tube. In an unsaturated soil, outward movement of water from an initially pure water reservoir, reduces pressure inside. The equilibrium value of this pressure is equal to the matric potential of the soil. The pressure in the tube can be measured in a number of ways, including a Bourdon gauge, or a pressure transducer.

Tensiometers are effective in the soil water range of saturation to -0.08 MPa. They do not operate in dry soils and at more negative potentials there is a tendency for air bubbles to develop. It is usual for de-aired water to be used, obtained by boiling or leaving in an evacuated container. However even so dissolved air can move in through the porous cup and come out of solution within the instrument, under the reduced pressure there. It is usual to "purge" tensiometers at regular intervals by replacing trapped air with de-aired water. During this procedure the suction within the tensiometer is released and some of the water can pass through the cup into the surrounding soil, so that readings taken after shortly after this are unreliable.

The response time of tensiometers can be variable, if they are not maintained in situ. Time is needed for water to move from the soil into the tensiometer or vice versa. Ceramic tensiometer cups have a response time, in the absence of any trapped air, of about one minute. The rate at which water can move is influenced by the conductance of the porous cup, and by the unsaturated hydraulic conductivity of the soil, while the amount of water that must move for a given change in potential, the gauge sensitivity, depends on the type of pressure sensor. Pressure transducers are more sensitive than Bourdon gauges, although the difference is lessened because of

the presence of air in the pressure transducer. Temperature can also cause changes in the readings of tensiometers.

1.5.3 Porous material sensors

The use of this type of instrument relies on the fact that their water content varies with matric potential in a reproducible manner. A physical property of the material varies with water content and is related to matric potential with a calibration curve.

In a saturated soil mass, water movement occurs as liquid flow through pores in response to gravity. A porous material in contact with this soil will absorb the soil solution by capillary flow.

As water is removed from this soil, the large soil pores empty of water and matric potential element is measured by the porous material.

As the moisture content of the soil falls below field capacity, the remaining water is bound to particle surfaces by adhesion. Moisture movement into the porous material is mainly by vapour diffusion vapour diffusion and the osmotic potential due to dissolved salts is added. The porous material measures total matric and osmotic water potential.

In summary, at high moisture contents, the matric potential is measured, whereas at low moisture contents, the total water potential is measured by the porous material method.

Methods based on the weight of filter paper, and electrical conductivity are discussed below. The physical properties of the material determine the range of matric potentials over which the sensor can be used. Sensitivity depends on the rate of change of the water content of the material with matric potential, hence on the pore size distribution of the material. The material itself may also display hysteresis characteristics. In addition the uniformity of sensors may be variable.

Filter paper method

The filter paper method uses changes of weight of the paper when in equilibrium with the soil water (Gardner 1937). Originally the paper was soaked in a sugar solution of known osmotic potentials and the paper which did not change weight was considered to represent the water potential of the soil. This method was altered to a paper soaked in salt solution and calibrated for weight versus osmotic potential. A final modification involved the omission of the salt, and this is commonly used in modern studies (Williams and Sedgley 1965).

This is a suitable method over the range -0.001 MPa to lower than -30 MPa (McQueen and Miller 1968). This is suitable for soil studies in arid regions, but drier than experienced by temperate plants. This is also a wider range than other methods. It has an accuracy of 2% moisture content at above -0.1 MPa. At lower water potentials, variability is less (McQueen and Miller 1968). However it may require calibration against six different methods of soil water measurement to obtain the wide range of soil water potentials.

The filter paper method also has a number of limitations. The soil sample for measurement should be greater than 100 g, as absorption of water by the paper would otherwise significantly affect soil water content. This method is either used with a soil sample in a sealed container, or used in situ in the soil. Even small disturbances when inserting and removing papers can change the potential of the cut surface. Greacen *et al.* (1987) attempted to minimise this disturbance by the use of access tubes into the soil. Filter paper has also been found to have a measurable hysteresis. Uniformity in response between equivalent pieces of filter paper is greater at low moisture contents (Gardner 1937).

Electrical resistance

This method has been used since 1940s. The resistance of the porous material is a function of its water content, and hence its matric potential. There are two common types of resistance sensor, gypsum and fibreglass sensors. The response of fibreglass and gypsum sensors is slightly different. Gypsum response is relatively small from saturation (0 MPa) until about -0.03 to –0.08 MPa, but then gives a rapid

approximately linear response as the soil dries further. Fibreglass sensors have a more rapid linear change in response than gypsum, from saturation to about –0.1 MPa. Fibreglass sensors are more sensitive than gypsum in wet soils, but the response is more uniform in gypsum blocks in dry soils (Aitchison *et al.* 1951, Perrier and Marsh 1958). Both of these sensors can be used in drier soils than tensiometers.

Air trapped in the sensor during wetting can alter its resistance, giving rise to hysteresis effects within the sensor. Resistance on drying is less than that on wetting a soil. Calibration based on a drying curve may give an overestimate of matric potential on rewetting, but since laboratory studies usually involve drying cycles, this error may not be important.

The resistance of the material is related not only to its water content but is also a function of temperature, and the concentration of solutes. The measured resistance Rs at temperature Ts can be converted to the resistance Rc at the calibration temperature Tc using the approximation

$$Rc = Rs[1+0.03(Ts-Tc)]$$

or

$$Rc = Rs^{[1+0.002(Ts-Tc)]}$$

The response time of the sensors depends on the unsaturated hydraulic conductivity of the soil, the contact with the soil, and the properties of the sensor. The rate of response to change in soil water is most likely to be important after rewetting of a soil by rain or irrigation, when there is a rapid change in soil water content. On drying, a sensor in situ in the soil, is in equilibrium with that soil water content and responds to the decreasing soil water.

The accumulation of solutes within the porous material leads to calibration drift, where a standard soil water content no longer gives the same resistance reading. This is particularly a problem in gypsum sensors. In addition they can be dissolved in highly acidic or saline soils. Calibration drift has also been observed in fibreglass sensors (England 1965). These were related to the deposition of soil colloids within the sensor and to decomposition of the fibreglass, over a period of three to 15 years. This can occur before any obvious signs of wear are visible.

Fibreglass and nylon are also sensitive to the electrical conductivity of the soil, which means they are less accurate. It is necessary in their design to minimise the contribution to the current between the electrodes from electrical conduction through the soil.

1.6 Effect of osmotic and matric potential on fungi

Micro-organisms differ in their ability to remove water from soil. With a soil-borne plant pathogenic fungus matric potential would dominate growth while the fungus is in non-saline soil. When it enters the plant root, osmotic potential would become important. Changes in response of the fungus to water availability could be due to either of these components, and should be tested in laboratory studies.

Cook *et al.* (1972) adjusted the osmotic potential of an agar medium for fungal growth with various concentrations of sucrose or inorganic salts. The sucrose or salt solutions were also added to oven-dried soil. In order to vary the matric potential, pure water was added to soil to achieve a range of matric potentials from a moisture release curve. Soil water potential was inferred from measurement of the equilibrium relative humidity using a thermocouple psychrometer. This work showed different responses in growth to osmotic and matric potential in one fungal species but not another. It suggested that some fungal growth responses may not be solely due to water availability but to solute availability, since many organisms have a preference for particular concentration of solutes. In a similar way Adebayo and Harris (1971) altered the osmotic potential of an agar medium with KCl or sucrose. The soil water content was maintained at a constant level, while varying osmotic potential was achieved with solutions of different concentrations of KCL or sucrose. The matric potential was altered with different volumes of water. Three different soil textures were also compared in this work. The osmotic and matric potentials for optimum growth were similar but the fungi were less tolerant of decreasing matric potential than decreasing osmotic potential. Therefore solute transport was considered to be also important in fungal response. There was no growth response to changes in soil texture.

If water potential is controlled by osmotic mechanisms, then a decrease in water associated with increased solute concentration could lead to possible nutrient imbalances and specific ion effects, as well water availability effects on growth.

If water potential is controlled by adsorption or surface tension phenomena, that is by matric mechanisms as is the case in soil systems, then decreased water potential is associated with decreased water content with the possibility of restricted solute transport.

1.7 Water in plant tissues

A large proportion of the water in mature plant cells is in the vacuole. This is separated from the external medium by the thin layer of cytoplasm, plasmalemma and tonoplast, which act as a semi-permeable membrane. The external medium outside is the water in the cell walls and intercellular spaces or apoplast. The solute concentration in the apoplast is low, so that the osmotic potential is small. The water potential in the apoplast is thus largely determined by the matric potential, which is influenced by the cell walls. Matric potential is high (>-0.1 MPa) in a well hydrated cell. This is one which has a large supply of water and is not losing much though transpiration, such as at night.

In the vacuole, there is a higher concentration of solutes than the apoplast. The osmotic potential is more important in determining total water potential. Matric potential is of less importance. Typical values of water potential in the vacuole might be -0.5 to -3.0 MPa. The water potential of the vacuole is less than that of the apoplast, because of the dissolved solutes. Thus water tends to flow from the apoplast into the vacuole, across the cell membrane. This raises the vacuolar water potential by diluting the solution in the vacuole. It also increases the volume of the vacuole. This increase would continue until the difference in water potential was zero, or the cell burst. However the volume of the leaf is limited by the cell wall in mature cells. Thus hydrostatic pressure increases in the vacuole, pressing the cytoplasm against the cell wall, and so raising the vacuolar water potential. In cells this hydrostatic pressure is known as turgor pressure. It is responsible for the maintenance of the leaf and eventually all the unlignified tissues in a firm or turgid state. At full turgor the

conditions in the cell are optimum for growth and function. Ultimately equilibrium is reached when cell water influx and efflux are equal.

It is customary to assume that the water potential of the cell cytoplasm is equal to the vacuolar water potential, since it is in equilibrium with it. The components of this water potential are not necessarily the same. Cytoplasmic matric potential may be a more important component because of the presence of colloids in the cytoplasm.

1.8 Mechanism of water transport in plants

Water flows from the soil through the plant to the atmosphere as a result of decreasing water potential. It is well established that evaporation of moisture from the leaves drives the movement of water upwards through the plant. In terms of water potential, water moves along a gradient of potential from the soil to the root, through the xylem to the leaves and atmosphere. A column of water can be maintained intact over the distance from soil to leaves due to cohesion between water molecules, a theory developed by Dixon and Joly (1894). The columns of water persist in the water transport vessels, because of cohesion between water molecules and adhesion with the walls of the tubes. Transpiration is loss of water form plants in the form of vapour. Transpirational loss of water from the leaves establishes the gradient of water potential in the soil/plant system. The water potential in the xylem vessel of a root axis falls below the water potential in the soil pores adjacent to its surface, then water flows into the root and passes via the xylem to the point of transpiration. Removal of water from the soil by plants is thus driven by the lowered water potential of the xylem and leaves. It continues until water can no longer be drawn from the soil. For temperate crop plant this corresponds to a soil water potential of -1.5 MPa from pores wider than 0.2 μm. Below this plants are generally unable to withdraw any more water, although the soil still retains water. This level is known as the permanent wilting point because plants will begin irrecoverable wilting at this stage.

Plants do not draw water only from the immediate vicinity of their actively-absorbing roots. As extraction of water proceeds, a depletion zone forms, which causes water to flow from the bulk soil to the root surface. There is a reduction in the

hydraulic conductance of the soil as it dries. As a result changes are needed in the plant root to maintain a steady flow of water to the root. This is achieved by progressive lowering of the root xylem water potential, and an increase in rate of lowering of water potential as drying continues.

1.9 Water uptake by a root system

Because of their perennial nature the root systems of trees have received considerable attention in their distribution in the soil. The root system of a tree is required to support its weight and to take up water from the soil. The form that a root system develops as it fulfils these functions was considered by Coutts (1987) in terms of the number and size of component branches. Soil exploration for water requires a finely divided root system, but for support the tree requires a few thickened roots at its base. The development of a root into part of the supporting structure is strongly influenced by its diameter. Large diameter roots have a greater chance of undergoing secondary thickening. In turn the diameter of the root is determined by the size of the root primordium from which it developed. The primordia are produced behind the root apex. The number and size of the primordia are governed by supplies of nutrients and growth regulators, in particular auxins. Root systems of trees consist of roots in all stages of development from delicate, newly formed unsuberized roots less than 1mm in diameter to old woody roots with a diameter of many centimetres. There are wide variations in permeability to water. The contribution of different roots in the root system to the uptake of water is not clear. Considerable absorption may occur through the older suberized roots (Kramer and Bullock 1966) as these may make up a large proportion of the root system. Up to half of the water uptake by the main axis may be supplied by older suberized regions further than 10 cm from the root tip (Sanderson 1983). When the lateral roots are included 75% of the total water transpired by the plant may be supplied by these suberized roots. The rate of water uptake of older roots is only approximately 10% of that by unsuberized regions, but the surface area of lateral roots is great enough to contribute greatly to the overall uptake. The response of zones of the root system to an increase in transpirational demand is greatest in mature root regions, particularly in the zone of lateral

emergence. The number of xylem vessels also increases with distance from the root tip, which increases the conductance along the root (Huang and Nobel 1993). The region of the axis where xylem vessels mature and become conducting coincides with the region where branches become active in water uptake (Varny and Canny 1993).

The response of tree species to changing water availability is often assessed by the root:shoot ratio. In humid zones, the water required for transpiration can be supplied by a relatively small volume of soil. Root:shoot ratios in these regions tend to be low; for example roots make up 21-25% of the total biomass of coniferous forests (Lange *et al.* 1976). In drier tropical savannah woodland the proportion rises to 30-40%, and 60-90% in some desert species. However the fraction of the root system which is active in water absorption may vary, and this is not accounted for in calculations of root:shoot ratios. The contribution of mycorrhizal tissues is also not included, nor increase in dry matter due to storage of carbohydrates or lipids.

1.10 Structure of vascular tissue

Two main xylem cell types conduct water within plants, vessel elements and tracheids. These cells have lost the majority of their internal structures to allow the flow of water with little resistance. A tracheid consists of a spindle shaped tube which is 3-5 mm long and originates from a single cell. A vessel consists of the cell walls of vessel elements connected end to end to form a hollow tube. The vessel length varies in different species of plants. Vessels and tracheids normally end obliquely with pits in these end walls which allow sap but not gas bubbles or particles to pass. The pit membranes are essentially the remains of the primary walls. The pit membranes at the end of the conduit cause resistance to the flow of water. Resistance to longitudinal flow is also dependent on the radius of the conduit. In most xylem conducting tissue there are two pits aligned from adjacent cell walls. Their most important role is to prevent the spread of vapour from one conducting vessel to the next when a vessel cavitates or is damaged. This system of two borders on the pit membrane gives low resistance to sap flow but the strength to resist collapse when there is a difference of pressure on each side of the membrane when cavitation occurs. The pores prevent the passage of gas bubbles, because of the surface tension of water which acts across the

pore. The shorter the average conduit, the greater the chance of water-filled conduits being adjacent to an air-filled conduit. The surface tension of water act on the bubbles trapped in the damaged conduits, which increases their internal gas pressure and induces them to dissolve in the water, restoring the conduit's ability to function. Therefore shorter conduits are more reliable in adverse conditions when cavitation is likely, in contrast with seemingly more efficient wider and longer conduits which are more vulnerable. Cavitation is more likely under drought conditions. The type of conduit varies between species. Following van den Honert (1948) a number of workers have estimated the relative sizes of the resistances to water flow of the different sections of the water pathway from root surface to leaf mesophyll, by treating the plant as a series of hydraulic resistances through which water flows in response to a gradient in water potential, in this way comparing it to Ohm's Law from studies on electricity. Studies of this kind tend to show that the root resistance is slightly larger than that of the stem or leaf, which is consistent with the idea that the pathway crosses living membranes only at the root endodermis. The root resistance is the largest component within the plant. However this simple model must be adjusted in a number of ways to make it applicable to plants in the field. Firstly, plants do not consist of a single root, stem and leaf in series. They should be considered as a number of root axes, branches and leaves attached in parallel to a stem, with each axis having its own resistance values. Secondly, water can be withdrawn into, or released from, storage in cells along the water transport pathway, altering the rate of flow. The extent of such changes can be assessed by measuring diurnal fluctuations in leaf thickness, stem diameter and root diameter (Kozlowski 1972). Thirdly, any treatment of transpiration should include water flow from the bulk soil to the atmosphere, but these cannot be fitted into the Ohm's Law analogy because the atmosphere is not at a steady state and the resistance of the soil is progressively increased as water is withdrawn. The resistance of the stomata is variable and there is evidence that the resistance of roots change as the rate of transpiration is altered. Also there is a change of state from water to water vapour at the leaf surface.

1.11 Mycelia of arbuscular mycorrhizal hyphae

The morphology of arbuscular mycorrhizal fungi have been extensively studied (Butler 1939, Mosse 1959, Nicolson 1959). Work by Friese and Allen (1991) has updated these. They provide a detailed account of the spread in mycorrhizal fungi in the soil. There appear to be three types of mycorrhizal inoculum, and two types of mycelia. The inocula include, spore-derived germtubes and hyphae. These germtubes are thin-walled (<1 μm) and 2-3 μm diameter. After primary contact of the germtube with the root, secondary contact is triggered, so that one spore may infect a root at several sites along its length, using a network of hyphae. The second type of inoculum is the runner hyphae. They have uneven wall thickness (1-3 μm) and diameter of 10-15 μm. They generally have few branches and either track along the roots causing secondary infections, or grow out into the soil matrix to infect other roots, giving rise to hyphal bridges. The runner hyphae seek out new sites for infection on the same or another root. They tend to cause single sites of infection on a root, in contrast to germ tubes. The third type of inoculum is derived from root fragments. The hyphae are thicker walled (3 μm) and diameter up to 20 μm. Vesicles do not seem to be required for initiating root infections, as root pieces with few or no vesicles are capable of infecting root tissue. Internal spread of the fungus appears to be limited (Allen 1982). External fungi initiate root colonisation.

The two types of mycelia extending out of an infected root are runner hyphae and the absorptive hyphal network. The runner hyphae are simple in structure and function. They are linear, non-branching, and appear to be solely concerned with further infection. The absorptive hyphal network is always made up of dichotomous branching hyphae, giving rise to a fan-shaped network, extending approximately 4-7 cm from the epidermal surface (Rhodes and Gerdemann 1975, Read 1984). The hyphae become thinner with each successive branch. Those near the root epidermis are thicker walled (>3 μm) and 10-20 μm diameter, the majority nearer 10 μm. By the 5th order branched the hyphae are <1 μm wall thickness, and 2-4 μm diameter. There is a correlation between branch order and diameter. The branching has not been shown to continue beyond an 8th order. This structure of the network may be limited by the flow of carbon from the plant root, and phosphorus inflow into the hyphae.

After the network has reached its full size, after 5-7 days, it dies back. There is likely to be the formation of a zone of phosphorus depletion at this maximum development (Pearson and Tinker 1975). This absorptive hyphal network allows the maximum exploitation of the surrounding soil volume, for nutrients and water. There has not been observed any role of the network in infection. The total hyphal network length per root entry point for fully developed networks with 8 orders of branching is 80-120 cm (Sanders and Tinker 1973). The fine thin-walled absorbing network develop septa as they lose their contents and become functionless (Mosse and Hayman 1980). This may have consequences for their ability to transport nutrients and water. As some networks die back, others form.

1.12 Changes in root resistance to water flow

There appear to be changes in root resistance related to the rate of water flow. A fall in resistance with increasing rate of transpiration has long been established (Jost 1916, Kohnlein 1930). There also have been reported diurnal changes in root resistance, with a minimum at midday, when water flow is greatest, and maximum near midnight (Parsons and Kramer 1974). A variety of mechanisms have been put forward. As the rate of flow increases, the contribution of osmotic and mass flow may vary (Fiscus and Kramer 1975), or the turgidity in the root cells is altered at different rates of water uptake (Brouwer 1954). Dehydration of cortical cells was also suggested as a mechanism for increase in resistance in the root by Huang and Nobel (1993). This decrease in conductivity during drought was thought to reduce water loss. There was rapid increase in conductivity after rewetting, enhancing water uptake. However this work was using two species of cacti, and may be a specialised reaction to arid conditions. Conductivity along the root axis was also found to be greater in older regions of the roots, farther from the root tips, because of the larger number of xylem vessels and their larger diameter. Loss in conductivity under drought stress was also found by Tyree *et al.* (1992) in a comparison of two tree species. Complete loss of conductivity occurred at different water potentials for the different species.

1.13 Influence of root resistance on plant water relations

The hydraulic resistance to the flow of water within the plant will not normally limit the rate of transpiration, but the resistance to water movement to the root from a drying soil can dominate plant water relations. In the presence of available soil water the rate of transpiration is largely determined by the stomatal resistance, whereas the hydraulic resistance of the plant determine the lowering of the leaf water potential that is required for water to flow from a soil at a given matric potential. Where air movement is low, the boundary layer resistance to gas exchange at the leaf surface strongly influences the rate of transpiration. The hydraulic resistance of the soil controls the rate of rehydration at night. This leads to a consideration of the resistances of the tissues of various tree species at a given soil matric potential and the role of roots in overcoming the problem of water movement in the soil towards the root, by changing the contact of the roots with soil water, by changing the distribution of the roots in the available soil.

1.14 Pathway of water flow

The pathway of water movement from the root surface to the site of evaporation in the leaf is predominantly extracellular (Spanswick 1976). In the apical 10-20 cm of young roots, water flows radially inwards through the cell wall and intercellular spaces of the root epidermis and cortex, up to the endodermis where further apoplastic movement is blocked by the Casparian strips in older root zones. Then water passes through the cells of the endodermis before entering the lumina of the xylem elements by way of the apoplast in the parenchyma in the stele tissue (Anderson 1976). Throughout its length the pathway to the leaves appears to cross membranes and pass through living cells only at the root endodermis. The water within most cells of the plant is not part of this pathway. The route of water flow is less clear for older and less permeable roots with a suberized exodermis, or which have undergone secondary thickening. The complex anatomy of roots results in a composite transport theory to explain changes in root radial resistance to water flow (Steudle and Peterson 1998). The are parallel apoplastic, symplastic and transcellular pathways for water passage from the soil, across the root, to the stele. There is a

rapid exchange of water between the parallel pathways. The large variability seen in the root hydraulic resistances may be explained by the use of the apoplastic pathway for water movement at high transpiration rates. At low transpiration rates, at night or during drought, the apoplastic pathway may not be used. This means that the water must flow through the symplasm or from cell to cell, where resistance is higher. This is supported by the finding that the radially resistance is approximately proportional to the amount of cortical tissue through which the water passes (Peterson and Steudle 1993).

Hydraulic flow of water is that determined by the gradient in water potential. However there are also osmotic flows because of the presence of membranes which limit solute passage. Osmotic water flow is negligible in the apoplast. The water flow across cells has a hydraulic and an osmotic component. The flows interact with each other while the water is moving radially into the root (Steudle 1994a,b). This interaction becomes important at low transpiration rates when hydraulic flow is lessened.

1.15 Alteration of water transport pathways in mycorrhiza

Mycorrhizal infection appears to alter water transport, but the mechanism for this is not clear. Safir *et al.* (1972) in a soybean-*G.mosseae* association demonstrated that mycorrhizal plants had 40% lower root resistance to water transport than non-mycorrhizal, by comparing the time for recovery of drought plant tissue. Mycorrhizal plants had higher shoot weight but similar root weights. Addition of a fungicide PCNB which inhibits P uptake did not affect the resistances. They suggested that hyphae could act as a low resistance pathway for water movement through the cortex into plants but since no effect of the fungicide was found this was discounted. However The differences in xylem structure in different plant species have also been suggested as explaining different responses of mycorrhizal plants to drought (Miller *et al.* 1997). The stele area of C4 grasses was almost all increased by mycorrhizal colonisation, whereas C3 grasses generally showed no change, and some showed decreases. These changes were also related to the responsiveness of the plants to mycorrhizal colonisation. A greater number of xylem vessels or an increase in the

vascular tissue allows greater transport of water (Fusconi *et al.* 1994). The resistances were found to be approximately equal in colonised and uncolonised rough lemon seedlings inoculated with *Glomus fasciculatum,* by comparing leaf potential and tranpirational flux for the mycorrhizal and non-mycorrhizal plants (Levy and Krikun 1980). Hardie and Leyton (1981) found lower resistances in mycorrhizal red clover than uninfected plants, even when the resistance per unit length of root is calculated, not the total length, which is higher in mycorrhizal plants. Kothari *et al.* (1990) found increased rates of water uptake per unit root length and per unit time in infected plants. The increased root resistance of mycorrhizal plants was also shown in rough lemon and *G.intraradices* (Levy *et al.* 1983). Root resistance was calculated from water flux related to fine root length and root water potential. They also suggested that the higher root densities and higher transpiration rates of infected plants depleted soil water more quickly leading to more severe water stress conditions during drought cycles. Reduced resistances might be due to the greater flow rates associated with increased absorptive surface area (Fiscus 1977).

1.16 Root system architecture

There have been many studies providing qualitative or quantitative information on the distribution of plant roots in soil profile in relation to soil water availability. These are normally concerned with the maximum rooting depth of the tap root, and the quantity of lateral fibrous roots which are produced under various environmental conditions. These were reviewed by Coupland and Johnson (1965). Fowkes and Landsberg (1981) considered the most efficient root system for water uptake. In conditions of adequate moisture a network of fine roots provides the largest surface area for uptake from the soil, but when this volume of soil has been exploited, fewer longer roots are needed to make use of water sources farther from the tree. A model by Fitter (1985) suggests that in high soil moisture content, root growth is topologically random, but at low soil moisture, this system is altered to a herringbone pattern, more suitable for soil exploration. The ability of trees to alter their rooting pattern has been shown in many studies, including in olive trees under changing conditions of soil moisture by Fernandez *et al.* (1991). At rain-fed sites root growth

could be at some distance from the trunk, but in drip-irrigated sites in drought conditions it was limited to the area close to the trunk. Where the soil was flood irrigated the root growth was uniform throughout the whole area occupied by the tree. This adaptability appears to be partly inherent and partly influenced by environmental conditions, since some species appear to be more likely or able to alter their root system under changing conditions of soil moisture (Callaway 1990, Arbez 1971). Water stress in Radiata pine causes reduced transpiration and growth, including root growth (Squire *et al.* 1987). Water stress appeared to cause a reduction in root extension rather than initiation of new roots. Root growth is greatest when soil moisture is maintained at field capacity, and is reduced during soil moisture deficit. Becker *et al.* (1987) found growth and survival to be highly correlated with moisture deficit in red pine. There were also decreases in the number of active mycorrhizal root tips. In a study of the response of root systems of two year old Scots pine and Sitka spruce to water deficit (Bartsch 1987), the spruce was less able to respond to a period of drought than the pine, and both species were influenced by the acidity of the soil. On a dry site, many roots die back in Manna ash and European hop-hornbeam, but are replaced by new growth (Abdul-Hadi and Zupancic, 1984). Most shoots were found to die in the first 10 years but could be replaced by new growth from dormant and adventitious buds. It was concluded that root biomass allowed this shoot regeneration. Drought has been found to affect root growth and shoot diameter growth, but only slightly height in Mediterranean firs (Becker 1977). Conditions in a particular year affect the girth increase, because cambial activity and carbohydrate storage in the roots continue after shoot growth has ended. However the height increase is not affected in that year.

1.17 Influence of mycorrhizal colonisation on the root system

Mycorrhizal plants show increased specific root length (m root g^{-1} root dry weight). They also show changes in the pattern of branching. The pattern of branching is rarely considered in studies on the water relations of mycorrhizal plants, because of the laborious nature of root architecture measurements. It has consequences for their water and nutrient uptake from the soil. It has been shown that mycorrhiza increase

the distribution of the root system in the soil, by penetrating smaller pores than roots, and by altering the architecture of the root system, so that it becomes more highly branched (Atkinson *et al.* 1994). This allows more thorough utilisation of available resources in a given soil volume. Decrease in plant dry weight with soil drying is greater for non-mycorrhizal plants (Bethlenfalvay *et al.* 1988). Plants colonised by AMF deplete soil to a greater extent than non-mycorrhizal plants, suggesting lower permanent wilting potentials for AMF plants. Berta *et al.* (1990) used *Glomus* sp. E3 in onion to assess changes in the branches pattern of the host due to the presence of the fungal partner. As shown in other studies, the root/shoot ratio was decreased by mycorrhizal colonisation relative to the control. However this difference was examined in more detail. The mycorrhizal root system was more highly branched with higher numbers of adventitious roots, and each branch was shorter relative to control plants. The rate of increase in growth of the mean total root length was greater in mycorrhizal plants. This alteration in the root system was caused by changes in the apical meristems of the mycorrhizal roots. The proportion of inactive apices increased rapidly in mycorrhizal plants compared with controls. Premature senescence of the root tip was induced by the fungus.

The effects on root branching of nutrient availability and mycorrhizal association were distinguished by Hooker *et al.* (1992). Poplar were inoculated with one of *Scutellispora calospora, Glomus* sp.E3 or *Glomus caledonium* in fertile soil conditions. There was no difference in plant size usually associated with mycorrhizal colonisation compared to uninoculated plants, but the root system morphology was altered. Mycorrhizal roots were more highly branched, than non-mycorrhizal. The number of secondary and tertiary branches was also positively related to the percentage mycorrhizal colonisation of the roots.

Alterations in root architecture are related to the host dependence on mycorrhizal association. Where the host is dependent on the association for growth, root branching can be increased by the symbiosis. Specific root length (m root g^{-1} root dry weight) of cool-season grasses was not altered by mycorrhizal colonisation with *Glomus etunicatum* but that of warm-season grasses was (Hetrick *et al.* 1991). Specific root length of cool season grasses was higher than warm season grasses,

which suggested that their roots were thinner in diameter and longer. These cool-season grasses were also less dependent on mycorrhizal symbiosis for survival. In mycorrhiza-dependent grasses, the specific root length decreases on colonisation. It was suggested that this was to conserve resources once the nutrient absorbing relationship was established. Root branching was inhibited by mycorrhizal association in warm-season grasses. Cool season grasses were more branched than warm-season grasses.

1.18 Water deficit

Stress has been defined as 'any environmental factor capable of inducing a potentially injurious strain in plants' (Levitt 1980). There is no one index of water supply from the environment to plants to express the degree of water deficit stress, because it is influenced by so many factors in the soil, plant and atmosphere. It is conventional to use plant indices of water stress rather than environmental indices. Principally tissue water potential is used, but also relative water content, and these are measures of the extent to which tissue water content has fallen below the maximum water content at full turgor, at which conditions are optimum for growth and function. Responses to different degrees of water stress differ at the cell or tissue level, and between different types of tissue. The photosynthetic uptake of CO_2 by the mesophyll cells in the leaf is associated with water loss to the atmosphere, and loss of turgor. Thus the leaves of plants are exposed to a degree of water deficit stress daily during illumination. Coping with this water stress is an aspect of all plants, not only those growing in dry habitats. There are many reports on the effects of different degrees of water stress, reviewed by Hsiao *et al.* (1976). Expansion of immature cells, which is the basis of plant growth is the most sensitive to water stress. It will begin to decrease in rate as soon as water potential of the cell is reduced below zero. As water stress increases cell biochemistry is disturbed. Protein and chorophyll synthesis are reduced under mild stress of -0.5 MPa. Under moderate stress of -0.5 to -1.2 MPa nitrate reductase synthesis, stomatal opening and CO_2 assimilation are reduced. Under severe water stress below -1.5 MPa respiration is increased. Proline and sugars accumulate. There is also a reduction in xylem conductance.

The water deficit experienced by plants in a particular climate is likely to be especially severe for tree species. The most obvious difference between trees and herbaceous plants in terms of their water relations, is the greater distance over which water must be translocated. Because of their height and the structure of their leaf canopies, trees face particular difficulties in maintaining favourable leaf water relations. In addition their perennial nature means that they experience a wide range of water availabilities. For these reasons the influence of mycorrhiza in tree species is of particular interest in this study.

1.19 Tolerance and avoidance of drought

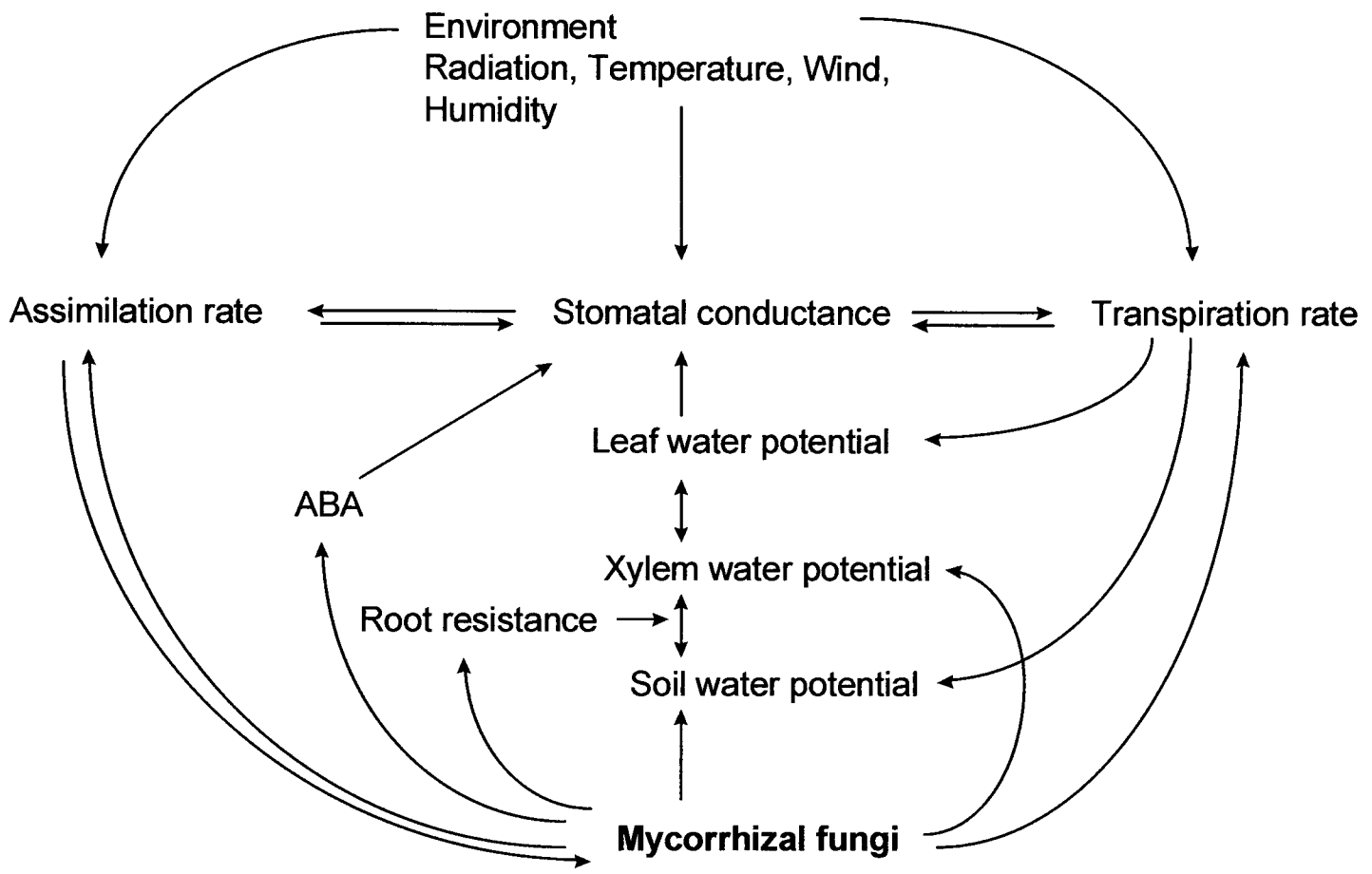
A distinction should be made between tolerance and avoidance of drought conditions. Both are mechanisms employed by plants. Plant attributes leading to the acquisition of the maximum amount of water are termed drought avoidance. These include root system morphology and distribution characteristics, such as increased root:shoot ratio, rapid root growth, plasticity of the root system, increased root branching, lowest sustainable root potential, osmoregulation in leaves-lowering of solute potential of expanding cell by accumulation of solutes especially sugars, amino acids and potassium ions. Attributes leading to the conservation and efficient use of water are generally termed drought tolerance. These are water use efficiency, that is the dry matter accumulated per unit of water transpired, differences between species in the canopy and leaf characteristics, leaf abscission during drought, stomatal response to humidity and to leaf water potential. However this may also be a drought avoidance response in the case of species which restrict activities to periods of water availability.

1.20 Mycorrhiza and response to drought

Some of the potential mechanisms involved in the influence of mycorrhizal associations on plant water relations are shown in Fig.1. These are discussed further in the sections below. The diagram indicates the relationship of stomatal conductance with the atmospheric environment and feedback loops with carbon dioxide assimilation and transpiration. It shows how the water potential in the plant and soil

are influenced by each other. The diagram also indicates the areas where mycorrhizal colonisation may have an influence on plant water relations. There may be alterations in the assimilation rate, transpiration (section 1.22), chemical signalling via abscisic acid (section 1.24), the resistance of the root system (section 1.15), and in the water potential of the soil and xylem tissue (section 1.17).

Fig.1 Possible areas of influence of mycorrhizal colonisation on plant water relations. (Adapted from Jarvis and Davies 1998)



This diagram was adapted by including the relationship of mycorrhizal fungi with the rest of the system. The overall responses to drought in mycorrhizal compared to non-mycorrhizal plants have been reviewed by Gupta (1991). In general arbuscular mycorrhizal plants show improved response to drought relative to uninfected plants, as defined by increased yield of above-ground plant parts. In soybean inoculated with *Glomus fasciculatum* grown in soil of high P concentration (58 mg/kg), mycorrhizal plants had a significantly lower root/shoot ratio than non-mycorrhizal plants in drought and non-drought conditions (Busse and Ellis 1985). Shoot weight was increased and root weight decreased in mycorrhizal relative to non-mycorrhizal plants. However the increase in shoot weight was not attributed to mycorrhizal enhancement of drought avoidance. It does suggest that resource partitioning was altered in

mycorrhizal plants. Transpiration, measured by weight loss, was similar in mycorrhizal and non-mycorrhizal plants. In wheat with the same mycorrhizal species or *Glomus deserticola*, the total plant biomass, the root weight and rooting depth were increased (Ellis *et al.* 1985). The increase was greater for *G.fasciculatum* than *G.deserticola*. In pepper inoculated with *G.deserticola* (Davies *et al.* 1992) drought tolerance was indicated by shoot water characteristics, such as potential, turgor, and content, which were greater in mycorrhizal plants. There was no significant difference in shoot weight with mycorrhizal colonisation, in contrast to Busse and Ellis (1985), but root weight and root/shoot ratio was decreased in mycorrhizal plants, in agreement. During low drought stress there was no difference in transpiration or leaf water potential. At high stress mycorrhizal plants had higher leaf water potentials and contents than non-mycorrhizal. Transpiration was unaffected by mycorrhizal colonisation. These specific changes in mycorrhizal plants are discussed below.

1.21 Influence of drought on gas exchange and tissue water potential

Stomatal conductance

The stomatal control of photosynthesis and transpiration has been reviewed by Jones (1998). It has been assumed that stomata provide the main short-term control of both transpiration and photosynthesis. Stomata operate in such a way as to minimise water loss relative to the amount of CO_2 uptake (Parkhurst and Loucks 1972). An alternative suggestion was proposed that the prime role of stomata was to avoid damage due to plant water deficit (Jones and Sutherland 1991).

There are feedback loops where changes in assimilation or transpiration rates resulting from changes in stomatal conductance can themselves affect conductance (Cowan 1972, Javis and Davies 1998). Feedbacks are either concerned with CO_2 uptake, either as internal CO_2 concentration or assimilation rate, or hydraulic feedbacks, which are dependent on plant water relations. In the hydraulic feedback loop, split-root experiments have shown that soil water status, rather than leaf water status, may have a controlling effect on stomata (reviewed by Davies and Zhang 1991). Stomatal conductance is generally presented as positively correlated to leaf water potential in published data, where the variable altered is the soil water status, in

drying experiments. However, there are infrequent reports where stomatal conductance is reduced (Morison and Gifford 1983), or unaffected by leaf water potential (Tardieu 1993). In these cases evaporation rate is altered, by changes in air humidity. This may account for differences in stomatal conductance between leaves or plants in a drought treatment.

Assimilation is not necessarily directly limited by stomatal conductance, particularly where photosynthetic saturation is achieved, by high light levels. This was demonstrated by Barradas and Jones (1996) when the stomatal conductance and CO_2 assimilation rate of *Phaseolus* leaves were compared at low (50 μmol $m^{-2}s^{-1}$) to high (350 μmol $m^{-2}s^{-1}$) photon flux density. Photosynthetic saturation was achieved relatively quickly, but the maximum stomatal conductance took twice the length of time to achieve. Thus when the plant was at its maximum photosynthetic capacity, CO_2 assimilation was no longer being determined by stomatal conductance.

Relative changes in transpiration and photosynthesis during drought stress

Dehydration of the soil first results in improved gas diffusion as water is replaced by air in the soil pores. Photosynthesis decreases as dehydration continues. When severely dehydrated net photosynthesis (when photosynthesis exceeds respiration) falls to zero. However leaf respiration is only moderately inhibited. So the production of carbohydrates is limited while the demand remains. The delaying effect of stomatal closure on leaf water loss has important implications for photosynthesis but not for transpiration. Gas diffusion is restricted by stomatal closure. Respiration can continue under these circumstances because oxygen is sufficiently abundant in the atmosphere to generate a large gradient in partial pressure in the inward direction, causing it to enter fast enough to compensate for stomatal closure. Carbon dioxide can diffuse out of the leaf in a similar way. During photosynthesis carbon dioxide must be supplied by diffusion from outside the leaf and the partial pressure is too low so that only a small gradient can develop as carbon dioxide is depleted into the leaf.

Plants differ in their photosynthetic response to dehydration, even when photosynthesis occurs at similar rates in hydrated plants. Differences in response can

also be found within a species, where the plants have been acclimated to drying conditions.

Under normal conditions, photosynthesis exceeds respiration. The surplus products of photosynthesis are available at night and to build non photosynthetic parts such as roots. When photosynthesis is inhibited but respiration continues the reserves of photosynthates is depleted. If this continues the lack of substrate for respiration causes decreased growth and eventually death of plant parts.

Photosynthesis can also be limited by a lack of substrates. Respiration often occurs when photosynthesis is limited (Huang *et al.* 1975a). Respiration generates water as a product so it seems unlikely that inhibition photosynthesis would be a direct result of lack of substrate water.

Carbon dioxide becomes limiting during dehydration. As stomatal closure is affect by dehydration, this limits diffusion of gas into the leaf. If the rate of photosynthesis is maintained at the same rate the carbon dioxide concentration within the leaf will quickly become limiting. However if the rate of photosynthesis is reduced metabolically, the requirement for carbon dioxide will decrease and there may be no shortage of carbon dioxide for the lowered metabolic rate. The effect of stomatal closure may then depend on the response of photosynthetic metabolism to dehydration.

Stomatal closure is also affected hormonally, by the production of ABA. It is normally present at low levels in leaves. In dehydrated plants the leaf ABA rises. The ABA rise is also correlated to stomatal closure. After rewatering stomata reopen but more slowly than the disappearance of ABA (Beardsell and Cohen 1975). Stomatal closure is closely related to the concentration of potassium ions in guard cells, and delayed reopening may be due to an inability to quickly recover the potassium ions (Ehret and Boyer 1979).

Recovery

The ability to recover photosynthetic capacity after a period of drought is important for resuming growth when water is resupplied. The ability to rehydrate depends on the extent of blockage of the vascular system (Boyer 1971b). The severity

of the dehydration also influences photosynthetic recovery (Fellows and Boyer 1978). Dehydration causes lysis of cells, as a result of membrane breakage, and this limits the number of cells which are able to recover when water is resupplied. There is also accelerated leaf senescence during dehydration (Boyer and McPherson 1975). This would also result in a decreased ability to recover photosynthetic capacity after drying.

Acclimation

Plants have been seen to acclimate to drying conditions, which suggests that they are able to alter their photosynthetic response to limited water availability. There is dehydration avoidance by the accumulation of sugars and amino acids in cells during dehydration (Crowe and Crowe 1986). These compounds contribute to acclimation through osmotic adjustment, and so delayed stomatal closure. There is also dehydration tolerance so that photosynthesis is less affected at a particular dehydration. The concentration of ions in the leaves, in particular magnesium, potassium or sodium, have been shown to influence the maximum rate of photosynthesis (Rao *et al.* 1987). Their concentration changes during dehydration.

Environmental factors

The changes in guard cell turgor that bring about stomatal opening and closing are dependent on a number of environmental factors, including light, carbon dioxide concentration, humidity and temperature (Schulze and Hall 1982), and on internal factors such as tissue water status and the level of plant growth regulators such as ABA and cytokinins. The effects of temperature on stomatal aperture vary among different plant species (Meyer and Anderson 1952). Direct effects of temperature can be confused with indirect effects due to larger vapour pressure deficits with increasing temperature. Temperature effects can be small in subalpine forest trees (Kaufmann 1982) or show increased stomatal resistance with increasing temperature (Wuenscher and Kozlowski 1971). There is interaction between light intensity and temperature (Pereira and Kozlowski 1977). Stomatal resistance is lowest at high light intensity. Stomatal conductance limits transpiration but rarely seriously limits photosynthesis

because this is usually limited by other factors (Farquhar and Sharkey 1982). Stomata usually open in the light but it is not clear whether this is a direct effect of light or because photosynthesis decreases the internal concentration of CO_2. Light does have a direct affect on stomatal aperture through photoreceptors in the leaf (Hsiao *et al.* 1973, Ogawa *et al.* 1978). The response of plants to increasing irradiance varies with plant species. There can be oscillations in stomatal conductance in cucumber with a period of about 20 minutes (Kitano and Eguchi 1992a), or wilting in direct sunlight, increased stomatal conductance initially and decrease after several hours in *Impatiens pallida* (Schulz *et al.* 1993). Where there is fluctuation in light intensity, which is usual in the natural environment, the stomatal conductance can also oscillate (Cardon *et al.* 1994). Oscillations in the carbon dioxide concentration of the atmosphere, such as those caused by air turbulence, can also cause oscillations in stomatal conductance.

1.22 Influence of mycorrhizal fungi on gas exchange

Stomatal conductances have been shown to be higher in wheat-*G.fasciculatum* and *G.mosseae* associations in wet and dry conditions than non-mycorrhizal plants (Allen and Boosalis 1983). Stomatal closure was at lower leaf potentials in mycorrhizal plants. Some showed no response even at very low leaf water potentials of -4 MPa. AM plants show lower xylem potential and higher leaf relative water content (Osonubi *et al.* 1991) Mycorrhizal maize inoculated with *G.intraradices* maintained higher leaf water potentials than uninoculated plants during drying (Subramanian *et al.* 1995) Stomatal resistance was lower in AM plants and transpiration higher.

During the hours of darkness, the transpirational demand for water is reduced. Water deficit in the plant tissues maintains the absorption of water from the soil. It is generally considered that leaf water potential is at its greatest just before dawn. It is also taken as being equivalent to the soil water potential, because during this period of reduced transpiration, the gradient between the soil and plant water potential lessen. Pre-dawn leaf water potential has been shown to be lower in mycorrhizal than non-mycorrhizal plants under drought stressed conditions, but the same under unstressed plants (Busse and Ellis 1985). Since pre-dawn leaf water potentials are in equilibrium

with the soil water potential, this suggested that soil water extraction was greater in mycorrhizal plants drought conditions. Under well-watered conditions mycorrhizal colonisation may lead to lower solute concentration in the root symplasm, resulting in lower root turgor (Auge and Stodola 1990). However following drought, mycorrhizal roots maintain greater turgor across a range of tissue moisture contents. This is due to increased partitioning of water into the symplast.

The leaf resistance to gas exchange was lower in AMF plants during drought stress but not when unstressed (Ellis *et al.* 1985). No difference in xylem water potential or proline concentration were found. No differences were found in transpiration, stomatal conductance or photosynthesis during drying, but all were higher in mycorrhizal plants during recovery after (Levy and Krikun 1980). Transpiration increased in AM plants (Levy *et al.* 1983) leaf water potential was lower in AM plants under drought but not when well watered due to changes in conductivity or soil water potential.

1.23 Mycorrhiza and nutrients

The effect of increased nutrient uptake and water uptake cannot easily be differentiated because many nutrients are in solution. Plants take up nutrients as inorganic ions from the soil solution. Substantial amounts of P may be held in organic form in the soil. Inorganic phosphates are only slowly released and tend to be poorly soluble in soil solution. Nitrates are very mobile in soil solution, as is potassium although required by plants in lower quantities. Phosphate and potassium uptake are particularly influenced by mycorrhizal colonisation (Harley and Smith 1983). Nitrates and calcium are less affected. Iron is bound in complexes with organic materials in the soil. Copper and zinc also tend to form complexes. Copper and zinc have been found to be more readily absorbed by mycorrhizal plants than non-mycorrhizal ones. Graham and Syvertsen (1984) found that flow of water to roots via the hyphae alone could not account for the greater water uptake by mycorrhizal roots. Conductivity of AM roots was higher, but phosphorus nutrition was mainly responsible for the greater conductivity of roots, since no differences were found between root hydraulic conductivity of mycorrhizal *G.intraradices* in citrus and non-mycorrhizal plants of

equal P status under well-watered conditions. Fitter (1988) suggested that the mycorrhizal effects on water relations of infected plants are a secondary consequence of the changes in phosphorus nutrition, due to changes in membrane permeability, but these effects are inconsistent. The effect of mycorrhizal infection on the drought tolerance of plants was studied in onion inoculated with *Glomus etunicatus* by Nelsen and Safir (1982) and also found to be associated with improved P nutrition. Despite P supplementation of non-AM plants they had lower P concentrations due to improved uptake in AM plants. This may be due to lower leaf osmotic potential, or greater concentrations of proline or other compounds.

P assimilation of drought stressed plants is improved with mycorrhizal colonisation (Busse and Ellis 1985). Even if P content in soil is high, its availability is low in dry soils, but mycorrhizal plants are better able to access it. Shoot nutrient concentration, in particular of P was lower in mycorrhizal plants relative to non-mycorrhizal (Davies *et al.* 1992) whether droughted or not. However non-mycorrhizal plants received a higher P fertiliser concentration.

1.24 Drought signalling: ABA as an indicator of stress

Stomatal closure, which leads to the water conservation and the avoidance of severe water stress, can come about by mechanisms other than enhanced carbon dioxide levels. Stomatal aperture can be reduced before changes in leaf water potential have occurred due to drying. This change can be in response to changes in water vapour content of the air, and is a sensitive mechanism in the control of the water content of the leaf (Losch and Tenhunen 1981). A further control of stomatal aperture is by growth substances in the leaf (Mansfield 1983). Abscisic acid is synthesised in the leaf choroplasts, and can cause stomatal closure and delayed opening. The abscisic acid concentration in leaves begins to increase at about -0.5MPa water potential (Hsaio et al. 1973). Root-shoot signalling appears to be more important in some plants than others. Plants have been grouped into those which tend to show a wide range of stomatal conductances for a small range of leaf water potentials by Bates and Hall (1981), and those plants, where stomatal conductance tends to be more closely related to leaf water potential (Fuchs and Livingston 1996).

Abscisic acid is synthesised by roots in response to soil drying (Davies and Zhang 1991). The signal compound is transported in the xylem to the leaves, where it alters stomatal conductance. However the exact relationship of this change is not clear.

A more recent suggestion for the mechanism of increased drought tolerance in mycorrhizal plants was put forward by Auge *et al.* (1995). The split root technique was used on sorghum infected with *G. intraradices, G. etunicatum* or uninfected. Three levels of phosphorus fertiliser, and two watering regimes, fully watered and half-dried, were applied. There was little difference between fungus species. The relative growth rate and leaf elongation were less affected by half-drying in mycorrhizal plants, than controls. The stomatal conductance was reduced in mycorrhizal plants. However, the leaf osmotic potential and relative water content were similar for control and half-dried plants. This suggests that the behaviour of the leaves of half-dried plants was not due to a direct hydraulic factor, but some indirect mechanism. The different levels of P did not affect growth or stomatal response of half-dried plants but did affect leaf length. It appears that mycorrhizal fungi can modify the leaf growth response to the root-to-shoot signal of soil drying, independently of the effects of plant size or P.

The production of plant hormones during drought has been shown to be altered by mycorrhizal colonisation. The reduction in cytokinin in stressed plants was less in mycorrhizal relative to non-mycorrhizal plants (Goicoechea *et al.* 1996). The concentration of proteins in shoots was similarly higher in mycorrhizal plants. It was reduced by drought, but less so in mycorrhizal plants (Ruizlozano *et al.* 1996). Mycorrhizal plants have higher concentrations of reducing sugars than non-mycorrhizal under drought conditions (Subramanian and Charest 1995). Isoflavanoid concentration is increased in mycorrhizal plants (Morandi *et al.* 1984) .

1.25 Mycorrhiza in growth chambers

The root and hyphal components of colonised plants have been separated in some studies, by allowing only the hyphae to grow through a mesh into a separate compartment (Faber *et al.* 1991, George *et al.* 1992), or by removing them under a microscope (Hardie 1985). These methods took account of the difference in size and

nutrient concentration in the tissues of mycorrhizal plants, so that the influence of AMF on water uptake could be made clearer. No evidence of significant direct water transport by AMF hyphae to the plant was found by George *et al.* (1992), but it was found by the other studies. Hyphal removal from colonised plants increased the root resistance by reducing the surface area for water absorption (Hardie 1985).

1.26 Aims

The emphasis of this study is on the influence of mycorrhizal colonisation on the water relations of tree species. It has been suggested above that plants are able to respond to changes in their environment using a number of mechanisms. The aim of this study is to elucidate the mechanisms of alteration in plant water relations with mycorrhizal colonisation. These include changes in water availability with time, or in spatial distribution. The concept of gradients in water availability which influence plant water relations is taken up in two different ways in this study. In Chapter 3 the response of mycorrhizal plants to changes in water availability with time are examined. In Chapter 4 methodology is tested to control soil water availability at specific levels. In Chapter 5 changes in water availability in spatial distribution are tested.

Three methodologies are tested. They include varying the soil water potential experienced by the whole mycorrhizal root system over time, and varying the soil water potential experienced by different portions of the mycorrhizal association. It is also intended to control the water potential experienced by the mycorrhizal root system and maintain it at a constant level.

Two mechanisms of altered mycorrhizal plant water relations are tested. The first involves changing the soil water potential to establish whether mycorrhizal plants are more effective at maintaining gas exchange at low soil water availability. Second, the relative importance of the external mycelium in water uptake is examined. Evidence for signalling of drought from hyphae to plant shoots is tested.

The literature review above indicates that the response to drought in mycorrhizal plants varies according to the association that is studied. Different species of plants have varying responses to water deficit. However those with similar anatomical structures tend to follow similar patterns. Similarly, the range in response of mycorrhizae to drought may follow patterns, with mycorrhizae forming groups of responses. The aim of the experiments in Chapter 3 is to compare the responses to drought of different associations. The hypothesis is that the response falls into groups of associations rather than a single response. A second hypothesis is that mycorrhizae

alter host drought response only at particular times during a water deficit period, and during the host lifespan.

The degree of water deficit imposed in mycorrhizal studies varies in the timing and severity of drying. The aim of the experiments in Chapter 4 is to impose a specific level of water deficit on mycorrhizal plants to develop a 'bench-mark' for mycorrhizal drought studies. The hypothesis is that mycorrhizal plants alter plant response to drought only at specific degrees of water deficit.

The distribution of roots and hyphae in the soil has important implications for water uptake. The relative distribution of external hyphae and roots must also influence water relations, in two ways. First, it influences the efficiency of the root-hyphal system for extracting water from the soil. Second, the proportion of the root system in a dry or wet volume of soil has consequences for its water state and response. If hyphae are considered as an extension of the root system, as they have been in nutrient studies, the relative proportion of roots and hyphae in a dry or wet volume of soil may give rise to different plant responses. This builds on the idea of root to shoot signalling of drought stress, depending on the degree and distribution of soil drying. The hypothesis is that hyphae in a different volume of soil from roots alter the host water relations not by extraction of water from these volumes and transportation to the plant, but by signalling of water availability in these soil volumes.

1.27 Objectives

The objectives of this project were;

To compare the water relations of tree species and their suitability for mycorrhizal studies.

To test the suitability of a gravimetric method for determining the transpirational water use of plants.

To control the water potential of the substrate surrounding mycorrhizal plant roots at a constant level.

To determine the suitability of nutrient solution culture for mycorrhizal studies, as a means of controlling the water potential of the plant growth medium.

To design and build a plant growth chamber, referred to as a rhizobox, to enable the separation of a portion of external mycelium from a mycorrhiza. This was to allow the independent study of the external mycelium.

To calibrate and install a suitable soil water measuring device in the rhizobox.

To compare the response of different mycorrhizal associations to changes in soil water availability.

To determine whether there is a systemic influence of mycorrhiza on plant water relations, or a direct contribution by the fungus to water uptake.

To measure the removal of water from soil by the external mycelium of mycorrhiza.

To determine whether water uptake by external mycelia is a passive or active process.

CHAPTER 2

Materials and Methods

This chapter details the plant material used in the experimental programme, together with general procedures used in subsequent chapters. The methods for production of plant material, soil preparation, measurement of root length, and mycorrhizal colonisation of roots are given here. Three different experimental systems were used in this study. Where methods were employed in only one experiment, these are detailed separately in the relevant subsequent chapter. Chapter 3 details the methods used in an experiment involving different fungal species in a host plant under drought conditions. Chapter 4 introduces a method for culturing mycorrhiza in nutrient solution. Chapter 5 describes the use of plant growth chambers known as rhizoboxes which separate the soil volume occupied by host roots and fungi.

Materials

2.1 Plant Material

Experiments were carried out using one of three species, Hybrid Black Poplar cv. Robusta (*Populus* x *canadensis* cv. Robusta), Wild Cherry (*Prunus avium*), and Field Maple (*Acer campestre*). These species were chosen as test subjects because they are commonly available from plant nurseries for woodland plantings. They also all form arbuscular mycorrhizae (Harley and Harley 1987). The plant material was obtained from a different source for each species.

The plant material used in the majority of experimentation, in Chapters 3 and 5, was hybrid black poplar *Populus* x *canadensis* cv. Robusta. This was supplied by Banff and Buchan Nurseries as one year "whips", unbranched hardwood stems. For the propagation of cuttings, the current years growth is the most vigorous. Poplars show little decline in the rooting ability of cuttings throughout the year. This allowed the propagation of cuttings throughout the year. Hardwood cuttings of 12 cm length with 5-6 buds were propagated from the whips. The cuttings were taken early in the morning, before drought stress was induced in the stems. This was in order to prevent wilting which would lead to a cessation of root growth. The lower end of the cutting

was cut between buds. The upper end was trimmed close to the topmost bud. These were planted directly into pots of soil for experimentation. As little as possible of the cuttings were left above ground to minimise loss of water by evaporation. Experimentation was begun once six leaves had been produced and had fully expanded.

Prunus avium was supplied by Horticulture Research International (Efford) as rooted micropropagated plantlets, in agar. These were planted into a mixture of 50% sterile potting mixture (Seed and Cutting Compost, John Innes, UK) and 50% 2 mm gravel, in black plastic pots (Desch pots 9x9x10 cm, LBS Horticulture, LBS Group, Lancashire). They were maintained under constant mist within a polythene chamber in a glasshouse, until the plants had produced at least one new leaf. This ensured that the plants were able to photosynthesize and so were no longer wholly dependent on the carbohydrate supplied by the agar. Once the plants were growing vigorously they were transferred to the pots of Craibstone soil. Experimentation was begun once plants had six new leaves.

Acer campestre was supplied as seed from Tilhill Nurseries. The seeds were put into moistened compost in a plastic bag and kept in a refrigerator at $1^{O}C$ for one month to stratify. Once a radicle had appeared they were planted in compost (John Innes) in seed trays and maintained in a glasshouse until they had two true leaves. At this point they were transferred to the Craibstone soil in similar plastic pots. Experimentation was begun once plants had six true leaves.

2.2 Soil characteristics

Two different soils were used for experimentation during this study. Both are from low altitude areas of Scotland. They were collected from topsoil on arable land. They were chosen for their textural and nutritional properties, and for the availability of data on their characteristics. Both Craibstone and Aldroughty soil are described in Appendix 1.

Craibstone soil is from the Countesswells series. This soil type is representative of a range of Scottish soils. It is also of medium texture with a range of soil particle sizes. Because of relative ease of collection, the availability of soil

moisture release data (Povey 1994), and its gradual release of soil water, it was convenient to use this soil type. The characteristics of Craibstone soil are shown in Appendix 1.

Countesswells Series is derived from a location near Craibstone soil and shows similar properties. Regression of soil moisture release data for Countesswells Series soil gave the following equations (Bache *et al.* 1981).

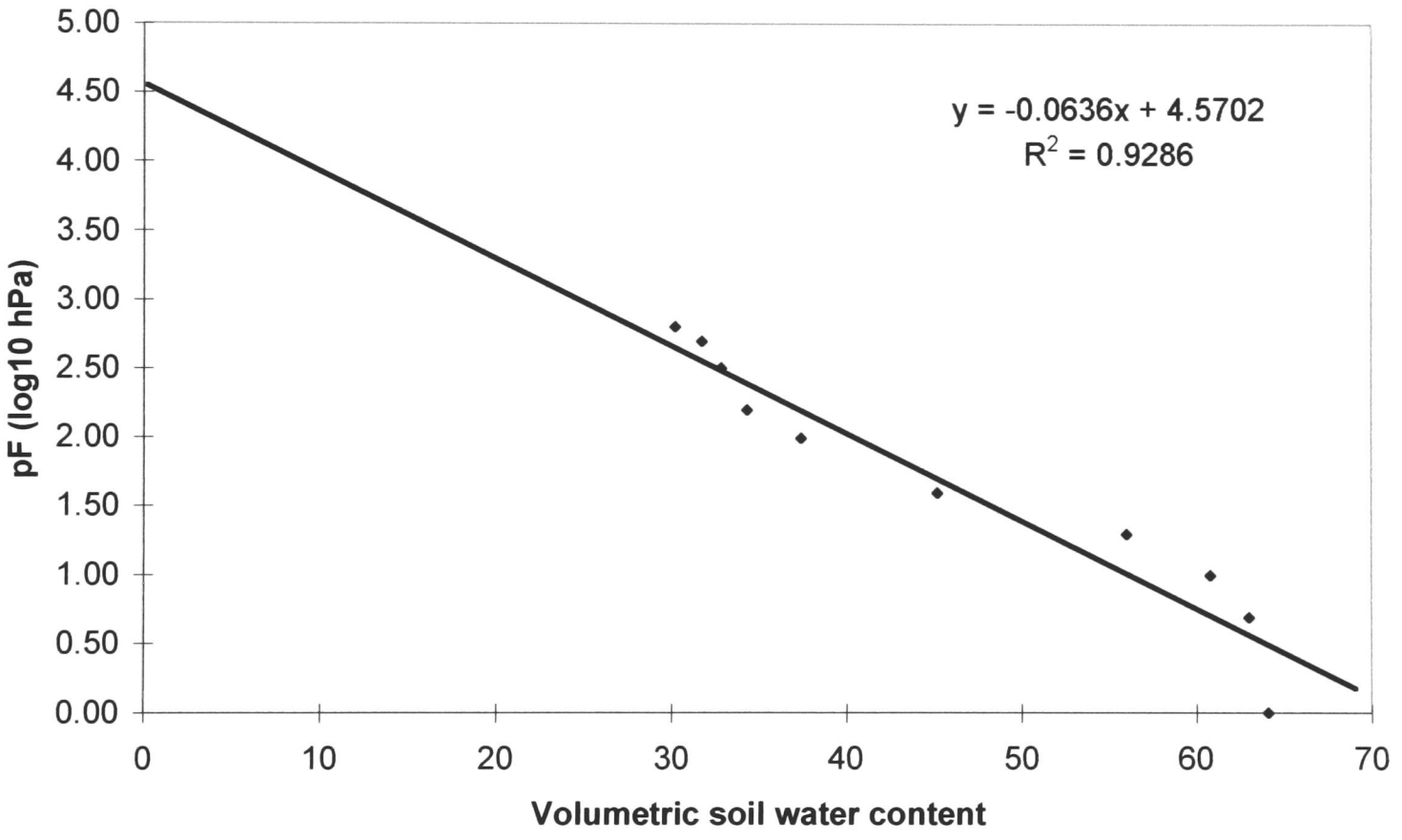
y = -0.0893 + 5.45 for Countesswells Series topsoil

y = -0.0833 + 5.03 for Countesswells Series subsoil

where y = log10 hPa soil water potential

and x = volumetric soil water content

Fig.2.1 Moisture release characteristics of Craibstone soil
Trendline was fitted by linear regression



Linear relationships fitted well for Countesswells Series soils and suggests that a linear relationship may also be used for Craibstone soil (Fig.2.1). The proportion of variation in $\log_{10}$ soil water potential data was well explained by variation in volumetric soil water content (coefficient of determination = 0.93). These two factors allowed the extrapolation of the regression to values of soil water content below those obtained empirically.

Soil from the Aldroughty area was also used for experimentation, because of its low organic matter content, to maximise the growth of arbuscular mycorrhizal fungi. It has been shown that the colonisation and growth of AMF is reduced by high concentrations of nutrients, particularly phosphate (Amijee *et al.* 1993, Bruce *et al.* 1994). In addition to its low phosphate concentration the Aldroughty soil was chosen because of its low clay content and less dense structure than Craibstone soil (see Appendix 1).

2.3 Soil preparation

The propagated plants used in experimentation were potted up in prepared soil in pots. In Chapter 3 these were Magenta pots (Magenta Plant Cell Culture Vessel, Sigmaware) filled with Craibstone soil. It was collected from the field and sieved to 3mm to remove stones. It was steam sterilised twice at 121^{O}C, 100 kPa for one hour in an autoclave (Midas 32, PriorClave, UK) to prevent weed or fungal growth from seeds or fungi present when collected. The pots were filled with soil to a bulk density of 0.82 g cm^{-3}. The weight of soil required to fill the pots to this density was calculated. A bulk density of 0.82 g cm^{-3} gave an oven dried weight of soil of 369.0 g per pot. The gravimetric soil moisture content of the collected soil was calculated by weighing to 0.1 g, oven drying at 80^{O}C overnight, then reweighing the soil to find the percentage water content. From this the quantity of moist soil required per pot to give the required dry weight was calculated and added to each pot. The moisture release characteristics of Craibstone soil packed to bulk density 0.82 g cm^{-3} are shown in Fig.2.1.

In Chapter 5 *Populus* cuttings were planted in specially designed rhizoboxes filled with Aldroughty soil. The soil was sieved to 4 mm. The sterilisation in an autoclave was again carried out. The sieved soil was packed to a bulk density of 0.64 g cm^{-3}, calculated from the dry weight of soil and the volume of the rhizobox. The design of the rhizobox is described in section 5.2.2. The cuttings described in Chapter 3 were miniaturised, growing to no more than 25 cm. The bulk density of soil in this experiment was therefore reduced to 0.64 g cm^{-3} in order to avoid any root

constriction which was a potential cause of this. Cuttings described in this section grew to be over twice the height of those in Chapter 3.

2.4 Growing conditions

All the seedlings were kept in a glasshouse with temperature control and supplementary lighting. Photosynthetic irradiance is the total energy falling on a leaf in the waveband 400-700 nm, measured in W m^{-2}. Photon flux density is the number of photons in this waveband, and is useful when related to physiological processes in photosynthesis. It is measured in E s^{-1} m^{-2} where E (Einstein) is one mole of photons. One mole of photons of a given wavelength carries 1.2 x $10^8/\lambda$ J, where λ is the wavelength of light. For daylight the relationship between photosynthetic irradiance and photon flux density is 1 W m^{-2} = 4.6 μE s^{-1} m^{-2} (McCree 1972). Bright sunshine provides 1000 W m^{-2}, and under 100% cloud cover the irradiance is similar to a plant growth chamber (Salisbury 1963). Two high pressure sodium horticultural lamps (Philips Son-T Agro) with power output of 400 W were used to light a glasshouse chamber in these experiments. A daylength of 16 hours from 06:00 to 22:00 was supplied. Plants were positioned randomly under the lamps so that all plants received similar irradiances. They were rearranged at intervals to ensure no preferential lighting for any plant or group of plants.

The growing season for experimentation extended from April to November. Photon flux density and air temperature in the glasshouse varied diurnally and seasonally, with temperatures between a minimum of 7^{O}C and a maximum of 32^{O}C, and photon flux density between 70 and 1000 μmol $m^{-2}s^{-1}$.

A fan (hydor-Tyro type TYR 1841B number B91, Donnington, Wilts. England) of diameter 450 mm was used to automatically cool the glasshouse chamber when the air temperature rose to 30^{O}C. It operated at 1250 revolutions per minute.

2.5 Preparation of fungal inoculum

Experimental plants were inoculated with root pieces colonised by specific mycorrhizal fungi. Stock cultures were kept to provide the inoculum. They were prepared in the following way. The original inoculum to make up stock cultures was

obtained from the International Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi (INVAM, U.S.A.). The species and origin of inoculum are shown in Table 2.1. It was in the form of dried rooting material montmorillitic clay containing root pieces, fungal hyphae and spores. This material was weighed and transferred to the soil medium beneath the prepared stock plant.

Table 2.1 Species of fungal inoculum

Fungal species	INVAM code no.	Origin
Glomus intraradices	FL-208-4	Florida,U.S.
Glomus mosseae	FL101A-1	Florida,U.S.
Gigaspora margarita	WV205A-5*	W. Virginia, U.S.
Gigaspora rosea	BR155B-1	Brazil

Only INVAM codes are presented since from 1996 there is no duplication of codes for cultures in INVAM and BEG collections (J. Dodd, personal communication).

A mixture of 1:1 of 1-2 mm silica sand washed in tap water, and seed compost was sterilised in an autoclave (Midas Priorclave) twice for 25 minutes. Plastic pots (Desch pots 9x9x10cm, LBS Horticulture, LBS Group, Lancashire) were cleaned with 50% ethanol, then rinsed with distilled water.

A trial of mycorrhizal host plants was carried out as part of ongoing studies within the research group. Cucumber was selected as the host plant for stock cultures because of its rapid growth and high production of pale roots in which mycorrhizal colonisation can be easily seen under a dissecting microscope. The seeds were surface sterilised in 5% sodium hypochlorite for 3 minutes and then rinsed three times in distilled water.

A mixture of spores, root pieces and clay substrate was put into the potting medium with the host seed. The stock cultures were supplied with deionised water according to requirement, so that there was no apparent drought stress but the rooting medium was not waterlogged.

When inoculum was required for the experimental studies, the host plants were harvested. Root sections containing large numbers of internal fungal structures

were identified at low magnification (x20) under a microscope (Leica Wild M10). The roots were cut into 1cm lengths. This inoculum was placed beneath the host plant when potted up. The quantity of inoculum supplied varied in each experiment and is detailed in each subsequent chapter. It varied between 0.1 and 1 g according to availability of mycorrhizal root material, but was always consistent within a group of replicate plants.

Methods

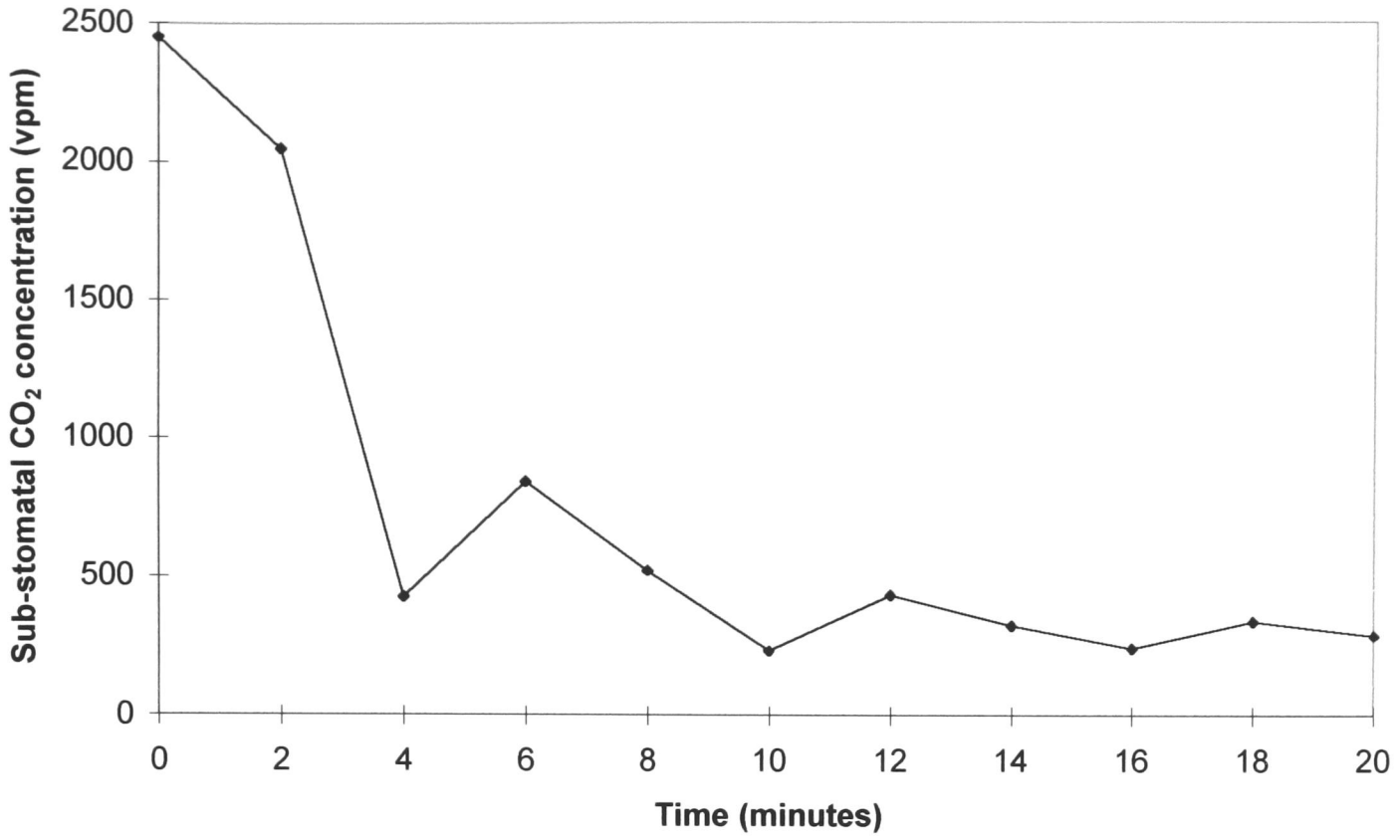
2.6 Measurement of Plant Shoot Processes

The gaseous exchange of the host plants was measured with a LCA4 IRGA (ADC, Analytical Development Company Limited, Herts.), carbon dioxide and water vapour infrared gas analyser. It is an open system with automatic pressure and temperature compensation, and carbon dioxide concentration is corrected for water vapour concentration. It was used in conjunction with a broadleaf plant leaf chamber. Measurements taken included change in carbon dioxide (mbar) and water vapour concentration (vpm) around the measured leaf, photosynthetic rate in μmol m^{-2} s^{-1}, transpiration rate in mmol m^{-2} s^{-1}, stomatal conductance in mol m^{-2} s^{-1}, and photosynthetically active radiation (PAR) in μmol m^{-2} s^{-1}.

The highest fully expanded leaf in the canopy was selected for measurement. Three more were measured at intervals down the stem. Only vigorous non-senescent leaves were chosen for measurement of gas exchange. In general the majority of the leaf surface was covered by the leaf chamber. Photosynthetically active radiation (P.A.R.) could vary during and between measurements, due to changing cloud cover. This variation could not be prevented but it was noted in the analysis of results, in later sections. Temperature variations were generally within 3°C during the course of measurements.

The length of time that a leaf was enclosed in the leaf chamber was kept to a minimum so that the atmospheric conditions around the leaf were as close to ambient as possible. However it was important that the leaf gas exchange should equilibrate. An indication of equilibrium was given by monitoring the calculated value for sub-stomatal carbon dioxide concentration. This value was dependant on stable water efflux. The initial unstable conditions in the leaf chamber resulted in readings of sub-stomatal CO_2 concentration which were very high. After a few minutes the readings were lowered and became stable, in the region of 100-500 vpm. At this stage a record of all the gas exchange parameters could be taken. Changes in the substomatal concentration of CO_2 are shown plotted against time in Fig.2.2. From these changes an equilibration period of a minimum of two minutes in the leaf chamber was chosen.

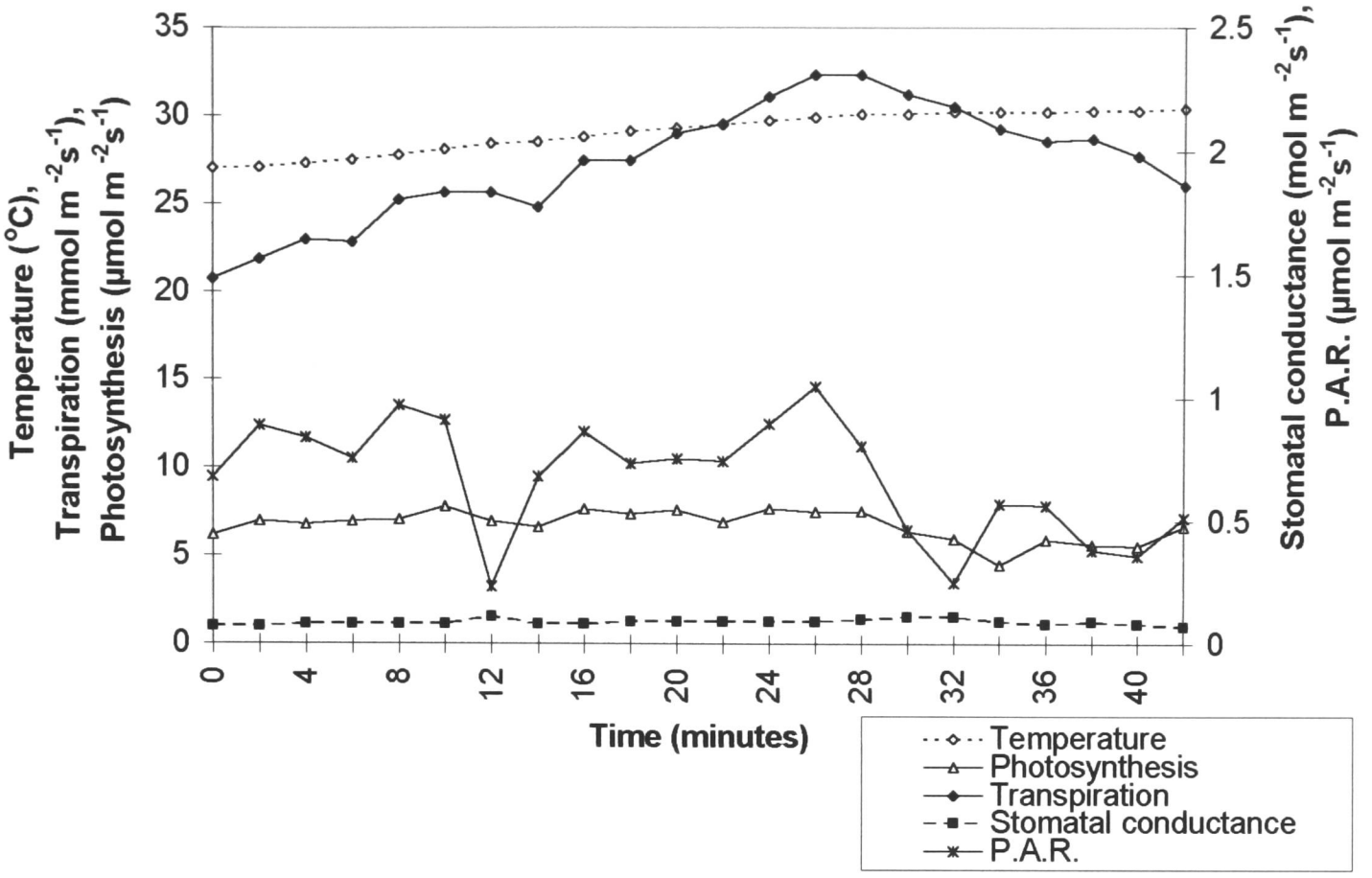
Fig.2.2 Stabilisation of sub-stomatal CO_2 concentration measured in an IRGA leaf chamber.



The limitation of time during which the leaf was enclosed in the chamber was also necessary to avoid increases in leaf surface temperature. Stomatal conductance was found to decrease slightly if enclosed in the chamber for more than 10 minutes.

Leaf gas exchange was monitored continuously for a 40 minute period to assess the effect of the leaf chamber on leaf processes if enclosed for an extended period (Fig.2.3). There was a small decline in photosynthesis and transpiration when the leaf was continuously enclosed in the IRGA leaf chamber. The leaf chamber temperature tended to increase by 4°C during this time. Transpiration reached a peak at 30°C and then declined, at a similar time as photosynthesis was reduced. Thus plant leaves were only contained in the IRGA leaf chamber for a maximum of 20 minutes continuously to avoid large increases in the temperature of the leaf.

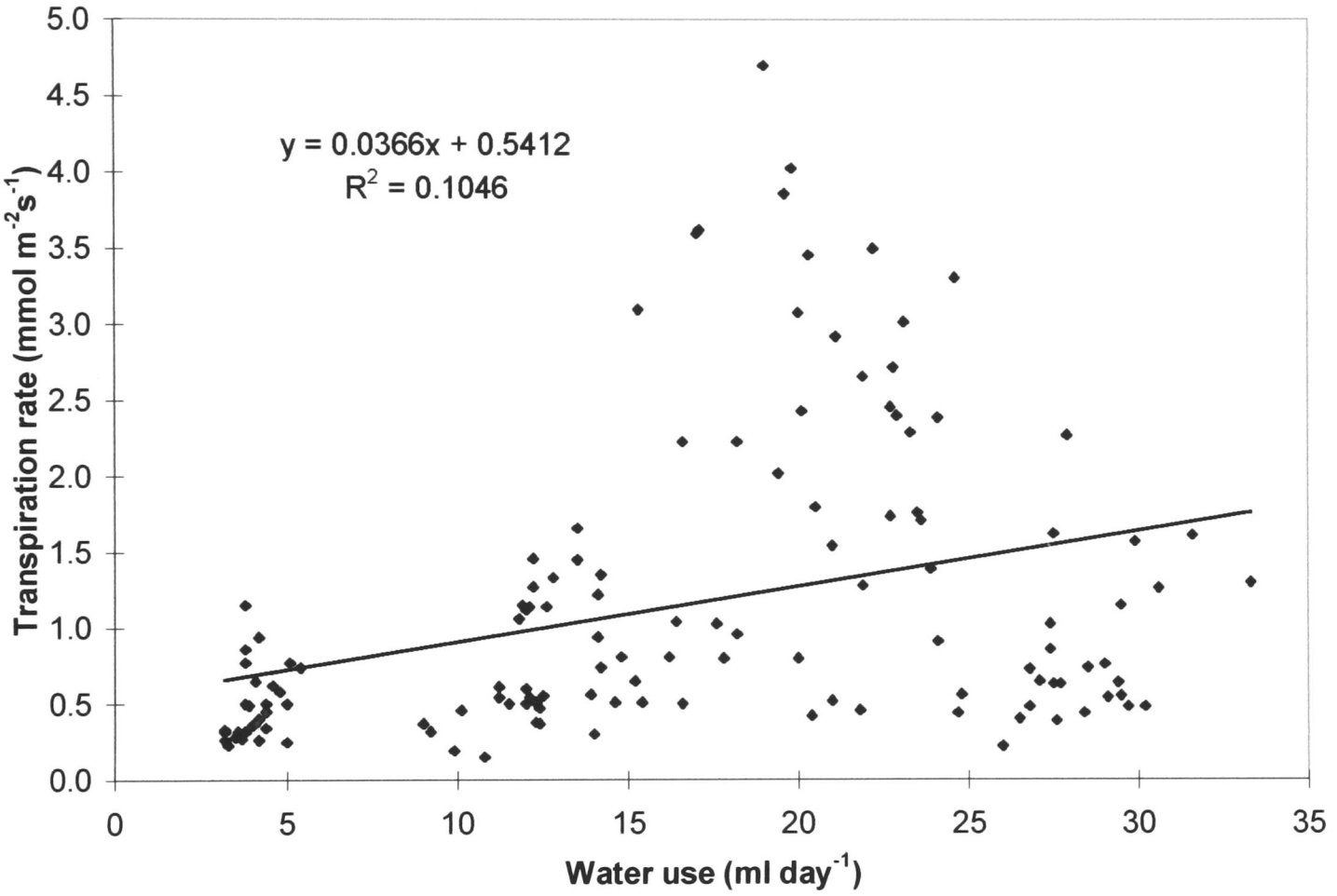
Fig.2.3 Gas exchange of one leaf when enclosed continuously in the IRGA leaf chamber



Measurements made with an IRGA can give differing measures of gas exchange from those obtained by other methods, such as gravimetric methods, because of the rapid air flow over the leaf surface generated by an internal fan in the plant leaf chamber. Air circulation in the glasshouse was maintained by an external fan (section 2.4). Water loss from *Populus* plants was measured using an IRGA and gravimetrically (described in section 2.7) to compare these methods. The calculated daily water use measured gravimetrically was compared with the value for transpiration measured using an IRGA on the same day (Fig.2.4). There was a significant correlation at 1% between transpiration and gravimetric water use. Generally the response of the plants followed the linear relationship, whether calculated from weighing or measured using the IRGA. However this relationship was not apparent on one sample day when transpiration measured using the IRGA, was higher than that expected from the linear relationship with gravimetric water use. There was a greater difference in transpiration with change in gravimetric water use on this day. In addition measurements made using an IRGA gave instantaneous readings, whereas measurements of water use measured gravimetrically were

averaged over 24 hours. Figure 2.4 demonstrates that the two methods are related, but IRGA measurements may overestimate transpiration. During daylight hours transpiration often exceeds water absorption from the soil, known as absorption lag. This is demonstrated in Fig.2.4 where transpiration greatly exceeded absorption in some samples.

Fig.2.4 Water use determine gravimetrically compared to transpiration measured using an IRGA
Trendline derived from linear regression



2.7 Use of the gravimetric method to measure the plant water use

This section details a trial to determine the value of the gravimetric method for measuring plant water uptake from soil. It also compared the water use of three species of trees to determine a useful model species for experimentation. The method used involved the estimation of transpiration rate by weighing a whole plant-soil system, and determining the change in weight between measurement periods. This method has been employed in drought studies on non-mycorrhizal (Jarvis and Jarvis 1963) and mycorrhizal plants (Busse and Ellis 1985, Levy *et al.* 1983). It is advantageous because of its simplicity and low cost. The method also allows large numbers of plants to be measured easily.

The gravimetric method determines the water content (θ) of the soil. This is calculated from the mass of water lost on drying a soil sample to a constant mass. The amount of water lost in drying increases with the drying temperature in soils that contain clay or organic matter, and therefore the temperature should be maintained at a constant level of $80^{O}C$. The water content is calculated as the fraction of the mass of water in the dry mass of soil. This is given as;

Gravimetric water content $\theta_{mass} = m_{wet}/m_{dry}$

It is usually expressed as a percentage of the dry weight of the soil.

Alternatively the water content can be expressed as a volume of soil.

Volumetric water content $\theta = \theta_{mass} \cdot \rho_{dry} / \rho_{water}$

where ρ_{dry} = density of dry soil

ρ_{water} = density of water = 1 M gm^{-3}

Volumetric water content is commonly used in soil water release curves, such as described above in section 2.2.

2.7.1 Limitations of the gravimetric method

The method makes several assumptions about the relationship between soil and plant. The first is that the soil water potential in the bulk soil is the same as that at the root surface. The soil water potential calculated from the weight of the pot may

not be that which the plant is experiencing, due to the decrease in hydraulic conductivity of the soil as it dries. As a result, this type of experimental procedure may not be suitable, where the root system of the plant is not uniformly distributed throughout the pot. It is probable that the plant is actually experiencing a more severe soil water potential if its roots are only extracting water from a smaller volume of soil.

In a large pot there will tend to be a lag in the movement of water through the soil to the soil/root interface, in comparison to the movement of water through the plant as it transpires. During the day when transpiration is at its greatest, initially water uptake will be from the area of the pot where there is the highest density of roots, which will also become the driest zone. At night when the rate of transpiration is slow, water will move from wetter zones and so will make a significant contribution, even if these zones are accessed by only a few roots. The plant water potential in general is thus influenced by the lowest soil water potential during the day, and the highest water potential at night (Kaufmann 1979, Campbell 1979). By using relatively small pots, and ensuring that root growth has occupied the total volume of soil, this problem can be minimised.

A further consideration with this method is that in a pot there is commonly a saturated zone in the lower portion. This gives rise to a situation where deeper roots have access to higher soil water potentials than roots higher up in the rooting zone. This leads to parts of the root system responding to different values of water potential (Rawlins 1979). This may not necessarily be a disadvantage to the method, since this situation occurs in the field.

This method of assessment assumes that the quantity of water stored in plant tissues during this period is negligible; this seems reasonable in small seedlings, and that the stored water component does not change during the measurement. It also assumes that dry matter accumulation is negligible during the period of measurement, or can be accounted for. Where these conditions are met then loss of water from the soil-plant system will be as a result of transpiration, which will be equivalent to root water uptake.

2.7.2 Purpose of trial

This trial aimed to compare the water requirements of three species of tree, without mycorrhizal colonisation. It represented preliminary work prior to further studies on the drought response of a single species with mycorrhizal colonisation. It was also useful to identify a model species for use in subsequent studies by examining water uptake between a number of tree species.

The criteria for the model species were;

-ease of availability from plant nurseries

-rapid water use to maximise the potential response to treatments in terms of water uptake. In order that the model system represented one where water was likely to be limiting, the selected species had to have a high water requirement.

-capacity to associate with arbuscular mycorrhizal fungi

-ease of handling for experimental work

-generalisations could be made about other related species

2.7.3 Plant preparation

Pots were planted with one of three species, Hybrid Black Poplar cv. Robusta (*Populus* x *canadensis* cv. Robusta), Wild Cherry (*Prunus avium*), and Field Maple (*Acer campestre*). These species were chosen as test subjects because they are commonly available from plant nurseries for woodland plantings. They also all form arbuscular mycorrhizae (Harley and Harley 1987). The plant material was obtained from a different source for each species.

Black 9 cm plastic plant pots (LBS, U.K.), square in cross-section, with a volume of 365 cm^3 to the inner rim were used. These are inexpensive, readily available, and of a suitable size for plants or cuttings of approximately 12 cm height. The pots were wrapped in polyethylene film. This film remains secure around the pot, even if wetted, and prevents water draining from the bottom. The film was secured about the stem of the plant with wire.

2.7.4 Soil drying treatments

The pots were subjected to three different moisture content regimes, which were equivalent to field capacity and two successively drier soil moisture potentials of -40 and -80 kPa, according to the soil moisture release characteristics shown in Fig.2.1. They were chosen because anecdotal evidence suggests that plants appear to respond to changes in soil water potential of half the soil field capacity. Field capacity is the percentage moisture retained by the soil when drainage ceases. Soil moisture tension at field capacity is commonly taken as being in the range -10 kPa to -30 kPa (Brady 1984). Here it was taken as -10 kPa. The corresponding volumetric water contents were calculated from the soil moisture release curve (Fig.2.1).

The water potential of the soil could be controlled by altering the water content of the soil. The volume of water added, which was required to achieve three soil moisture potentials was calculated from the soil moisture release curve, to give the volumetric water content. This was converted to gravimetric water content using the equations above, and found directly by weighing.

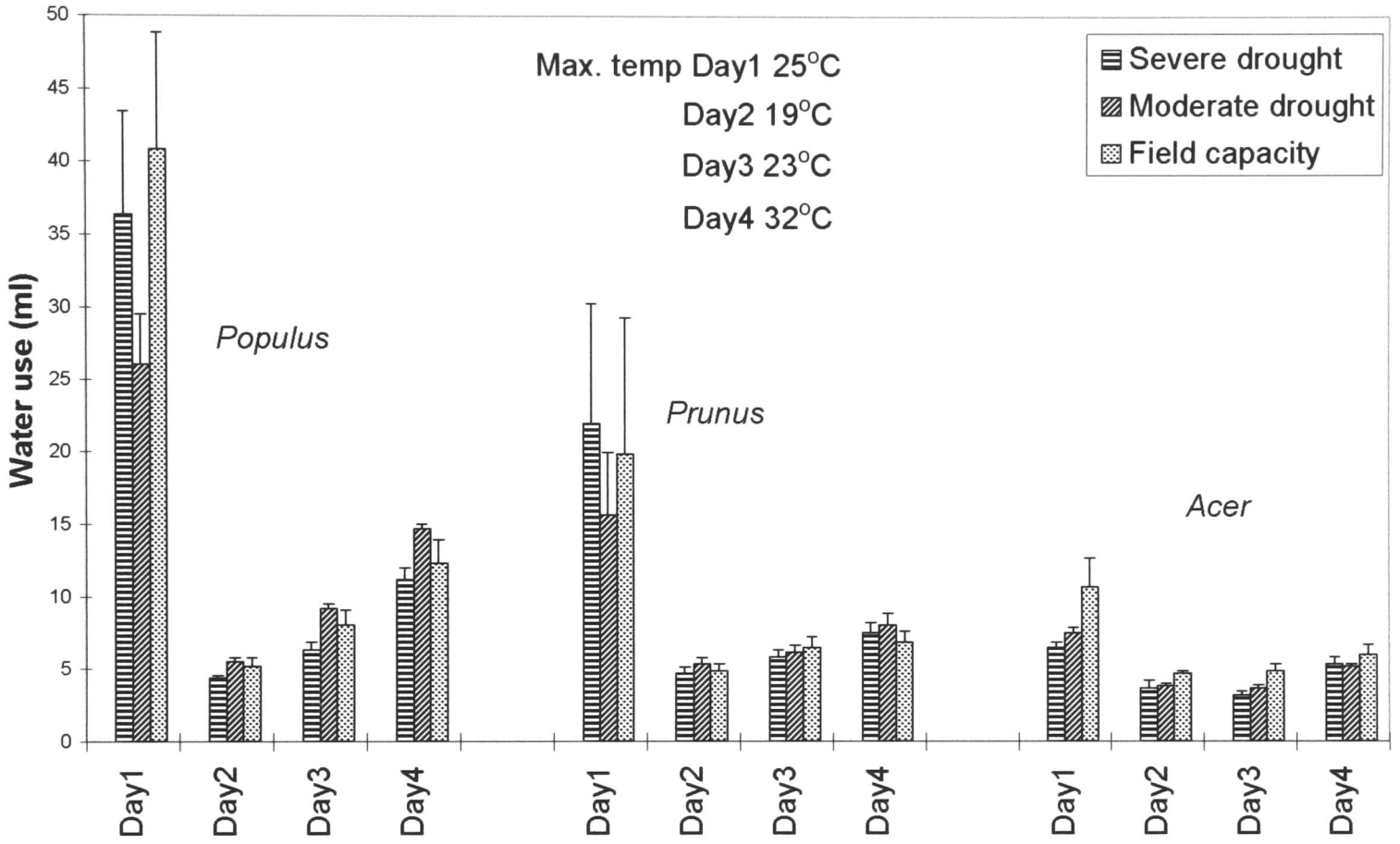
The pots were watered to the required moisture content and then subjected to repeated drying cycles. A drying cycle was continued until the plants were beginning to wilt. This generally occurred on the same day for all replicates, but not for all the species. All species were however rewatered at the same time. The quantity of water needed was found by weighing the pot. This cycle of watering and drying was repeated three times. Water use was monitored throughout, for a period of 17 days. At this stage in the experimentation the effects of hysteresis were ignored. The volume of water transpired daily per plant was calculated. These results are given in Fig.2.5.

2.7.5 Results and Discussion

This study was intended to determine a suitable model tree species for subsequent experiments on drought tolerance of trees with mycorrhizal associations, and to assess the suitability of the gravimetric method for further studies on plant response to soil drying.

There was much greater transpiration in all species on the first day of the drying cycle than on the following days. Transpiration decreased as water availability dropped after the first day of the drying cycle (Fig.2.5). This change could be distinctly shown by the reduced daily weight changes of the plant pots.

Fig.2.5 Daily water use per plant in three tree species
Water use measured gravimetrically



The transpiration was related to the maximum daily temperature. After the first day of the drying period, water use was greater when the air temperature rose.

There appeared to be some differences in the volume of water transpired between watering treatments, field capacity, moderate and severe drought, in *Populus* and *Acer*. These differences between watering treatments were not generally identified as significant using two way analysis of variance. On Day 3 however, watering differences were significant at 5%.

Populus had the highest initial use of water, followed by *Prunus*. Differences in water use between species were significant at 5%.

At the beginning of the drying cycle immediately after plants were watered, there was higher variability in water use than on the following days as indicated by the standard error shown.

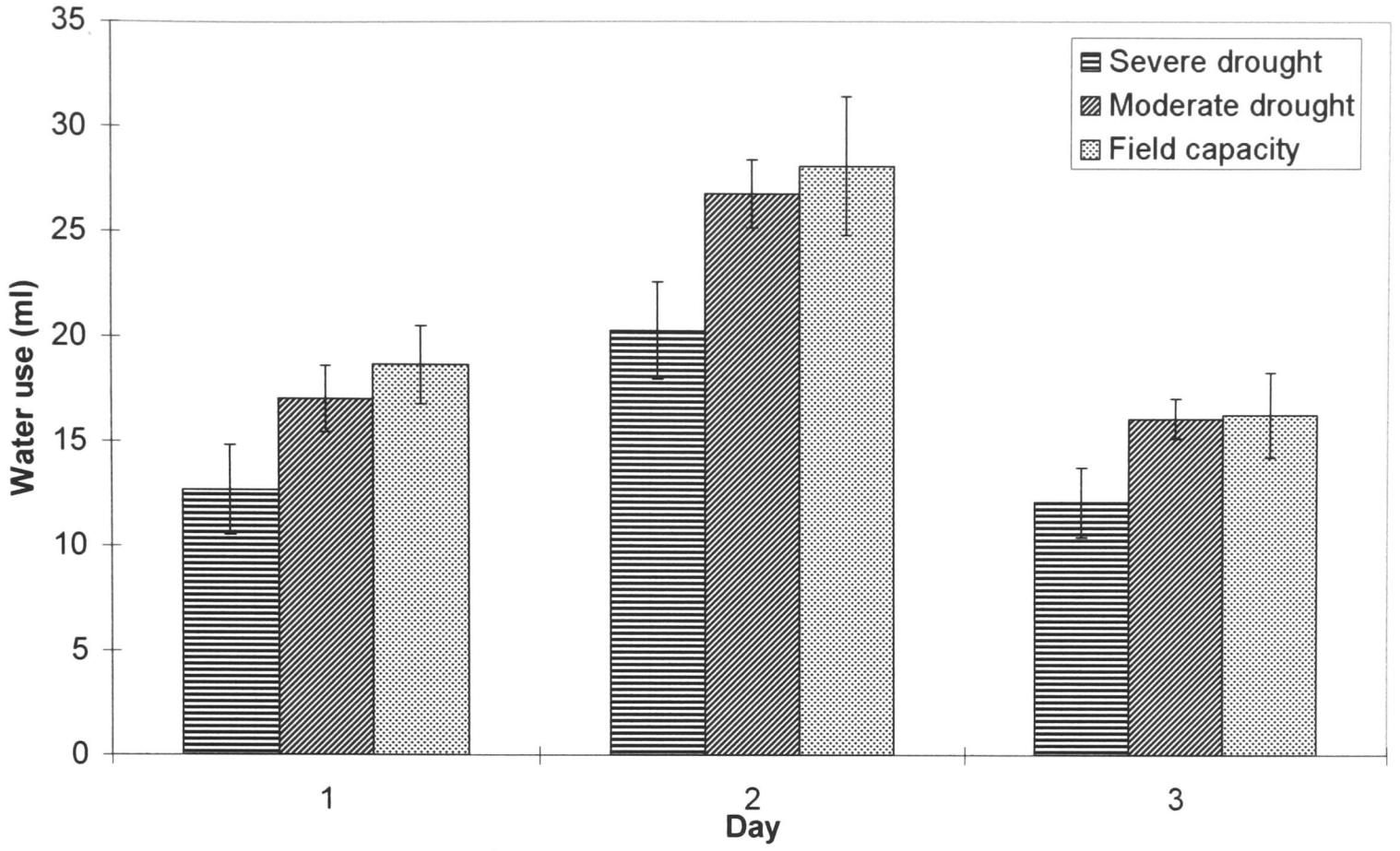
Analyses of variance showed significant changes in the weight of plant pots with time after water was withheld, and with the watering treatment. There were also greater weight changes at higher air temperatures. The fresh weight of the plant was approximately 15% of the weight of water in the soil, indicating that any change in water content of the plant was largely negligible relative to changes in soil water. These results suggested that weight change was following a pattern which would be expected in transpiration.

There were no interactions between species and treatment. This suggested that all species responded in the same way to the imposed treatments. Although this was unexpected given the differences in leaf shape and growth pattern seen between tree species, it was therefore possible to choose a model species on another basis. The species was chosen on the basis of ease of handling

Poplar was chosen for the majority of further experiments, because it was easier to handle cuttings rather than seeds or micropropagated plants. It also showed the highest water requirement. This suggested that it would give a more dramatic response to watering treatments and consequently allow experiments to be carried out faster. The method used in this preliminary study could be used to control and easily measure the water use of plants, in further experimentation.

When the drying cycle was repeated with *Populus* only (Fig.2.6), a significant difference was found in water transpired between watering treatments ($F=3.2$ $p=0.0015$).

Fig.2.6 Daily water use by *Populus*



The results of this trial suggested that the plant species used in experimentation could be chosen for properties other than their water use. Clonally propagated plants should show greater similarities in their development and response to experimentation than those derived from seed. In Chapter 4 *Prunus* plants were used. This was due to their small size, being micropropagated plants (see section 2.1). They fitted easily into laboratory glassware which was ideal for nutrient solution culture, described further in Chapter 4.

2.8 Measurement of soil moisture using electrical resistance sensors

2.8.1 Choice of sensor

The methods available to measure soil water have been reviewed in section 1.5. Those which were considered for the current experiments are noted here. The requirements of the chosen soil water sensor were determined by its use in a rhizobox, described in section 5.2.2, where changes in soil water would be measured. A device which could be left in situ with minimal maintenance was required. It was preferable if it could be sealed into the rhizobox to minimise evaporation.

The filter paper method was carefully considered as an inexpensive choice. It has been found to be accurate if good contact is made with the soil. However the mass of soil in the rhizobox was small (see section 5.2.2), and the potential for proportionally high losses of water during insertion and retrieval of the paper was great. It was also important to ensure that the hyphae developing in the soil were not disturbed, or damaged. If a filter paper method was used an adequate seal would have to be achieved when the paper was repeatedly put in place and removed.

Use of a tensiometer would have been the preferred method for use in this study, because of the direct measure of soil water potential. Manufacturers were consulted to find one which was small and adaptable for use in a rhizobox, but this was unsuccessful. Those commercially available at low cost are not easily adapted to use in a rhizobox, because of the positioning of the water column. The useful measurement range is also limited. Other methods such as thermocouple psychrometers were considered too expensive.

The requirements were adequately met by electrical resistance sensors. The adaptability of the various porous material electrical resistance methods for soil monitoring meant that they were the most appropriate method for use in subsequent studies. They were durable and of a suitable size for use in the rhizobox. The range in water content measured was large. Uniformity between sensors was also high. Water content is a less useful measure than water potential in describing plant water relations. However since information was available on the relationship between water content and potential, the resistance sensors were a suitable method for monitoring changes in soil water. A soil moisture and temperature sensor (ELE International), a

porous material sensor with resistance varying with water content, was chosen for the following reasons. With dimensions of 15x10x3 mm it could be easily inserted into both sections of the rhizobox. These sensors provided a soil moisture value as resistance and a temperature value from a thermistor reading. The rhizobox could be sealed with the connective wires extending from the sensor, and no further disturbance of the soil would occur. There was also the possibility of recording the data automatically using a datalogger. Finally the instrumentation and sensors were inexpensive so that sufficient could be purchased for many rhizoboxes.

2.8.2 Description of soil water monitoring equipment

Soil moisture and temperature sensors (ELE International) were chosen to monitor the soil moisture content of the two sections in the rhizobox. The soil moisture monitoring equipment consists of a soil unit which is positioned permanently in the soil, and a meter unit to which it is connected via three wires, and is used to measure the electrical resistances of the soil unit, to determine soil moisture and temperature. The soil unit consists of a moisture sensitive element and a temperature sensitive element. The moisture sensitive element is a "sandwich" of fibreglass cloth between electrodes. The electrical resistance between the electrodes, through the fibreglass, varies in response to changes in moisture content of the soil. The thermally sensitive element is a thermistor. These two elements are enclosed in a perforated metal case 3 mm thick. This thinness and its exposure to the soil on both sides ensure good capillary contact between the soil and fibreglass, and minimises the time required for water in the fibreglass to reach moisture equilibrium with that in the soil. The sensors were found to give a constant value after imposed changes in water content within 1 hour. However the advantage of this product is that in being in permanent contact with the soil, the sensor is subjected to the same continuous changes in water potential as the soil. The cases are die-formed to ensure their consistency and uniform compression of the fibreglass.

The meter unit is a battery-powered alternating-current ohmmeter, operating at 93 Hz. The alternating current is transmitted into either the moisture- or the temperature-sensitive element of the soil unit, chosen by the user via the control

panel. The current is passed through the soil sensor and then rectified for indication on a direct-current microammeter. The measurable electrical resistance range is 0 to 1.5 billion ohms.

2.8.3 Calibration of soil moisture and temperature sensors

When in equilibrium with the soil the moisture and temperature sensors can be used to determine the soil moisture potential, by relating the resistance readings to calibration curve of resistance versus moisture percentage for each soil tested. The sensors were to be used in plant rhizoboxes described in section 5.2.2. One of the rhizoboxes was used for the calibration. This was performed by inserting sensors in the test rhizobox with Craibstone soil packed to the same density as intended in the subsequent experiments in Chapter 5 i.e. 0.64 g cm^{-3}. The calibration involved two stages.

The first calibration carried out concerned the response of resistance to changes in soil water content.

The second calibration introduced a temperature element, to test the influence of variations in temperature on the resistance of the sensor.

2.8.4 Relating resistance to soil water content

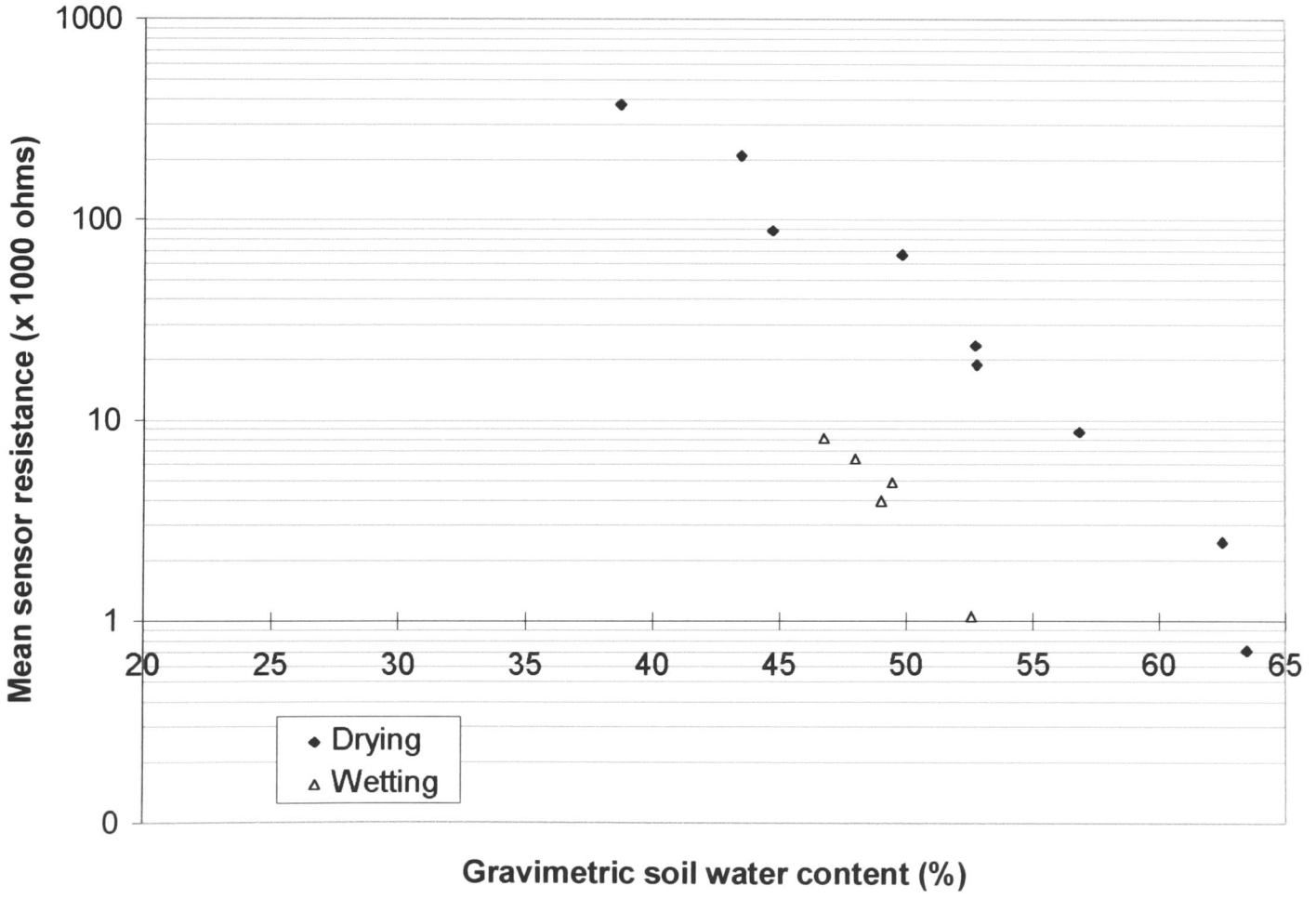
The resistance produced must be calibrated for soil water potential or content for each soil type that is used. Therefore a calibration curve was made up for the resistance sensors, where the soil was watered to saturation and allowed to dry by evaporation. Soil water content was determined by weighing the complete soil and container, at the same time as the resistance reading was taken from the resistance meter. The temperature was kept constant at 15°C in a controlled environment cabinet. A calibration of resistance produced for varying soil moisture content over wetting and drying cycles was carried out.

The uniformity between sensors has been found to rarely exceed 0.5% deviation in moisture content (Colman and Hendrix, 1949). This was tested by placing sets of sensors in the rhizobox, adjacent to each other and monitoring the measured

resistance of each sensor at a given soil water content. Differences in measured resistance were analysed for statistical significance by one-way analysis of variance.

The resistance of the fibreglass sensors was found to be related logarithmically to the water content of the surrounding medium. The resistance measured by the sensor is plotted against the gravimetric soil water content in Fig.2.7. There was a negative relationship between resistance and soil water content. Resistance decreases as the soil water content increases. The sensitivity of the sensor resistance was 100 ohms. Hysteresis effects were observed. When these were taken into consideration, wetting and drying cycles were considered separately. The drying cycle was used in further calculations of the relationship between water content and resistance. One-way analysis of variance was performed to assess sensor variability. There were no significant differences in resistance response between sensors ($F=0.02$, $p=0.9$), indicating that uniformity between sensors was high.

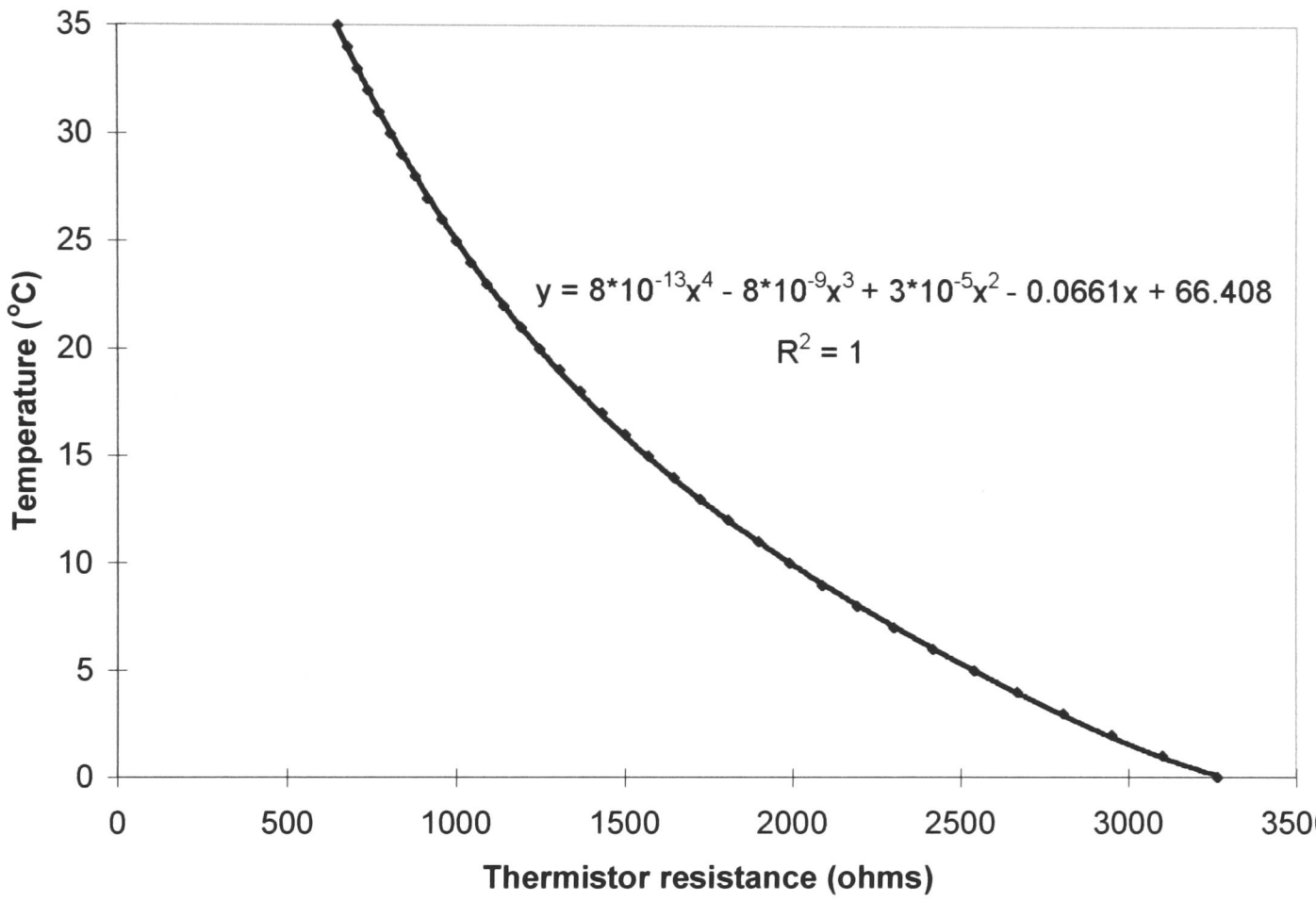
Fig.2.7 Response in sensor resistance to changes in soil water content on wetting and drying



2.8.5 Temperature adjustment

The temperature of the soil surrounding the sensor is measured using a thermistor. The response of the thermistor to temperature followed a polynomial equation. The change in thermistor resistance with changing temperature is shown in Fig.2.8.

Fig.2.8 Relationship between thermistor resistance and temperature



Temperature = $8*10^{-13}x^4 - 8*10^{-9}x^3 + 3*10^{-5}x^2 - 0.0661x + 66.408$

where x = resistance of the thermistor ($R^2=1$)

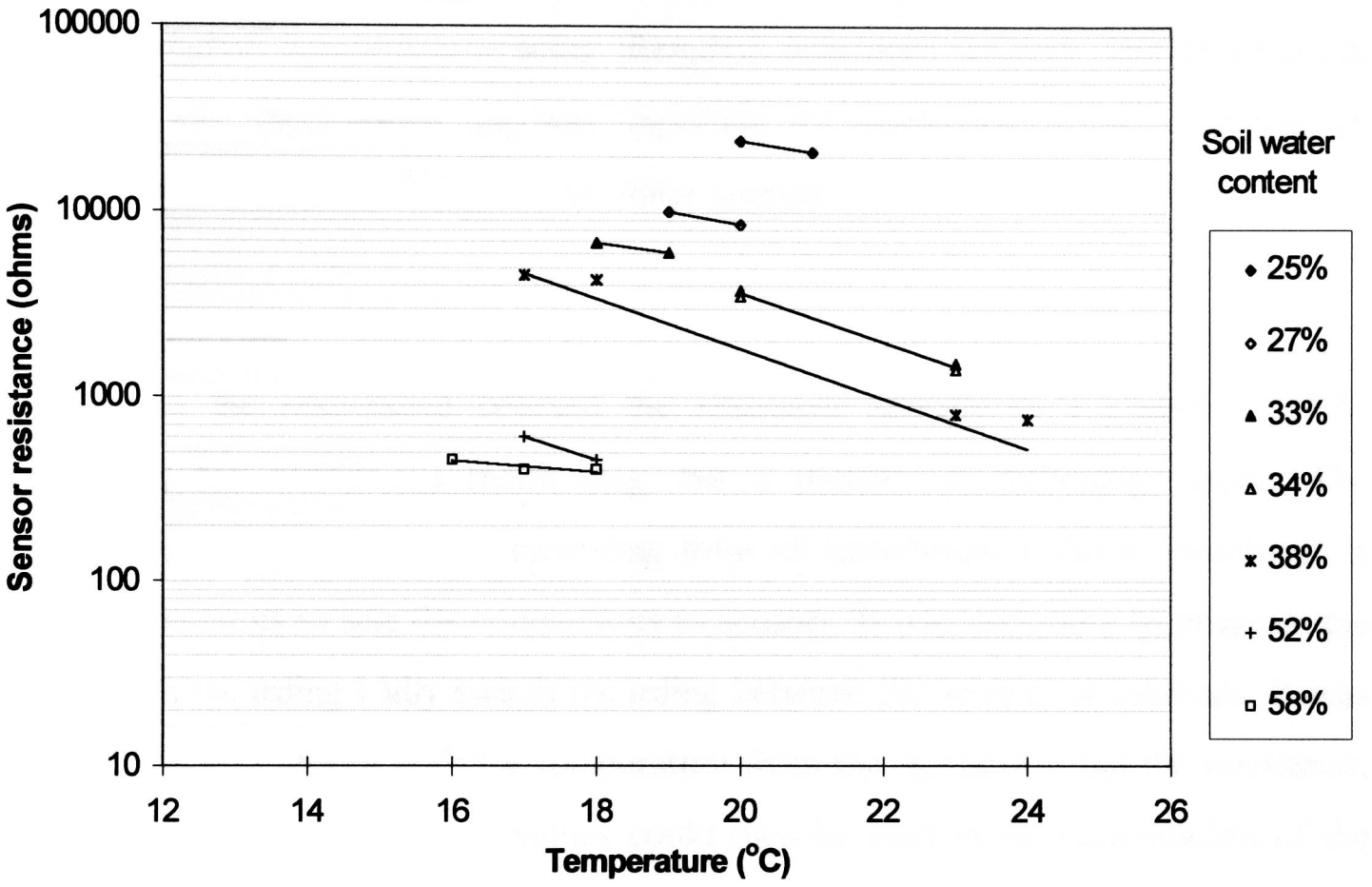
This temperature measurement was used in further calculations of water content. The resistance of the fibreglass/electrode sandwich is sensitive to temperature fluctuations. At higher temperatures the resistance is reduced. This gives a misleading impression of the wetness of the soil. A correction was thus required to provide the equivalent resistance at a standard temperature. This was carried out by measuring resistance and rhizobox weight at a range of temperatures for a given soil moisture content.

The rhizobox was put into a temperature controlled chamber. The temperature was varied and the soil moisture content varied by adding a known volume of water

or allowing the soil to dry. The resistance of the soil moisture sensor and temperature was measured and the moisture content of the soil found by weighing the rhizobox.

Fig.2.9 shows the variation in sensor resistance with soil water content at varying temperatures. The resistance of the sensor decreases at higher temperatures, for a given soil water content.

Fig.2.9 Variation in sensor resistance with soil water content and temperature



Linear regression was carried out using the logarithm base 10 values of resistance and those of temperature to relate to the gravimetric soil water content. Comparison of regression calculations of water content with and without the temperature variable showed that although the temperature variable made no significant change in water content value, it had a dampening effect on the variability in readings, and was therefore a useful measure in these water content readings. Although the changes were not statistically significant, there was a difference of 1-5 % in water content between these calculated values, those without a temperature variable being higher.

The equation of soil water content related to resistance and temperature was;

Water content(%) = 84.8178 -0.2953 x temperature -8.6861 x log_{10}resistance
R^2 = 0.96

	Standard error	p-value
Intercept	12.74	0.0012
Temperature	0.68	0.68
Log_{10} Resistance	1.10	0.0005

These calibrations showed that a temperature variable should generally be included in measurements of soil moisture using fibreglass resistance sensors. The temperature component of the measurement was important for smoothing variation. It was an extra variable in the calculation of soil water content.

2.8.6 Datalogging

It was attempted to connect the resistance sensors to a Campbell CR10 datalogger, for continuous monitoring, but a number of difficulties arose. The datalogger was not capable of recording from all rhizoboxes without investment in further hardware to make the connections to sensors. It was necessary to program the datalogger to systematically switch recording between the sensors at intervals. It was also necessary to record both the temperature from the thermistor and the resistance, for each data point. These two values could then be used in the computation of the water content, according to a mathematical relationship previously derived from calibration. It was not possible to write the program necessary in the time available. It would have been advantageous if data collection could be made with a datalogger but much more time was needed for adaptation of the system to a datalogger. It would be a useful addition to the method in further work.

2.9 Root length measurement using image analysis

Measurements of root length are used as a measure of the potential for water and nutrient uptake by the plant. Measurements of root length are laborious, even when partly automated using a computer. Root length per gram are also not constant, so that the weight can not always be directly related to the root length. This is particularly true of woody perennials, where root density and diameter alters with age. As specific root length (length to mass ratio of the root system) changes, the root to shoot ratio which optimises water and nutrient capture from the soil is altered.

Root length was measured using an image analysis system, the Quantimet 600 (Leica (Cambridge) Ltd., Clifton Road, Cambridge). This system uses levels of grey with a chosen threshold level to detect an image of a sample object via a video camera and display it on a computer screen.

The measured length of an object is used to calibrate the length of the image of the object on the computer screen. The system was calibrated initially, and before each day of measurements. A precise length of 20.00 mm was set between the pincers of electronic callipers. The image of the callipers on the computer screen has a length given in pixels. The actual length is entered into the Quantimet package, which calibrates the computer image. The conversion is then provided for pixels on the screen to units of length of the object. The limits of measurement of the callipers and the screen resolution determine the accuracy of the calibration. This finishes the calibration for a camera at fixed focus. However the system must be recalibrated if the video camera is moved or focusing is altered, as this alters the screen image. The accuracy of the calibration was checked by introducing wire pieces of total length 100 mm and finding the length using the procedure below. Accuracy was within 3%.

The procedure for analysing root samples was as follows. It requires the user to adjust the light intensity of the image on the screen. The first stage is "Image set-up". With a blank screen, i.e. no objects in the field of the camera, the light intensity of the screen is adjusted. At this stage supplementary lighting from a light box or overhead lamps can be introduced. The aim of this stage is to determine a background level of lighting which will give the greatest contrast between it and the sample. This is largely determined by the properties of the sample and is found by trial and error. In

this study with roots it was found that ambient lighting in the room should be reduced, and lighting of the image provided by a light box. In addition there is shade correction which allows any irregularities in the field of view, such as scratches or marks on the surface of the light box to be removed from the screen image. The level of lighting without the root sample is then accepted as the base level of light and is set up as the "blank field".

The sample is then returned to the field of view. The image is accepted by the computer using "Acquire". It is no longer a live image and is represented by pixels of varying intensity. Once the image has been acquired, it can be adjusted so that it most resembles the sample, before measurement.

When a sample is being measured the desired image must be selected from that shown on screen as regions of light and shade. This is done by selecting a threshold level of light which most closely covers the desired image. For this reason it is desirable to have clean samples with little adhering soil, and a bright background. The image is "detect"ed by altering this acceptable light level, until the "detected" image is the same as that perceived by the operator to be the desired root sample.

This then provides a basic image of the sample. However it requires editing in order to enable its length to be found. This is done using the "amend" menu. Unwanted sections such as soil can be "erase"d by drawing with the mouse. Sections of the sample which have not been selected, such as finer or paler roots, can be drawn in.

The detected image is then "skeleton"ised. That is, it is reduced to the width of one pixel. The actual width of the sample is disregarded and it is reduced to a series of lines of equal width of one pixel. This image can be "prune"d to remove any excess lateral branches of the skeleton which are not present in the root sample. This tends to occur when thicker roots are skeletonised and is a feature of the system.

The whole field of view is measured and the value of the perimeter of the selected images is given. Because these images are one pixel wide, half of this perimeter value gives the length, of the sample.

A difficulty in length measurements is encountered if the roots cross over each other. This is because at the crossover point the length of both roots is represented by

the same pixels in the image. These pixels are only included once in the calculation of length, so there is an underestimation of the total root length. Without further programming, this situation is best avoided by ensuring that no roots in the sample cross. It is this preparation of the sample on the viewing surface that is particularly time consuming, requiring up to 15 minutes per sample image.

The reproducibility of the measured length of the sample is largely determined by the accumulation of the errors in calibration, and image detection. These can be rather large in the region of up to 10% of the actual length of the sample. They are minimised by maintaining the same position and focusing of the video camera, and the same lighting levels on the sample and screen image.

The plant root system was subsampled by removing a portion down the central axis which included root from the base and tips of the root system. This was chopped roughly into pieces 1-2 cm long and a subsample removed to give a fresh weight of 0.2 g. The length of roots in one screen view was generally in the region of 1000 mm. Portions of the subsample were analysed until the subsample was complete. The roots were dried and weighed. The root length of the dried subsample was used to calculate the total root length of the whole root system from the total dry weight of the root system.

2.10 Root clearing and staining

Trypan blue is a commonly used stain for micro-organisms. Phillips and Hayman (1970) developed a procedure using trypan blue in lactophenol blue to show the internal structures of fungi clearly against the outlines of cells in the cortex of intact roots.

Roots were cleared using the method of Koske and Gemma (1989) which makes some modifications of the method of Phillips and Hayman (1970). The procedure was carried out in a water bath at 75-85^{O}C. The roots were kept in 2.5% w/v potassium hydroxide for at least 2 hours. This solution removes much of the cell cytoplasm so that the vascular bundle and root cortical cells are visible. The roots were then rinsed and put into alkaline hydrogen peroxide (20 ml NH_4OH in 80 ml H_2O + 90 ml H_2O_2 (30% solution) made up to 900 ml with water) to remove root pigmentation which took approximately 2 hours. The length of time for clearing was increased from the 15-60 minutes suggested by these authors because of the age of the roots and the high degree of suberization. Roots were acidified in 1% w/v HCl for 30 minutes. Acidification was necessary for successful absorption of the stain. They were then stained at 75^{O}C in trypan blue in acidic glycerol for a further 30 minutes. This timing was found to be adequate to stain hyphae and internal structure but leave the root epidermal cells pale.

This method permitted the storage of stained root samples for several months. The samples were stored in acidic glycerol in the dark. There was no decrease in stain intensity nor any growth of micro-organisms.

Fig.2.10 Stained root showing internal structures of *G.intraradices*.

2.11 Assessment of mycorrhizal colonisation of roots

Assessment of the level of mycorrhizal colonisation of host roots was carried out according to the grid-line intersect method. It was chosen because of the low standard error of this method in assessing root infection (Giovanetti and Mosse 1980).

The stained roots were spread out evenly in a Petri dish so that none overlapped. They were examined at low magnification (x20-25) under a binocular dissecting microscope (Leica Wild M10), with a grid in the eye piece. The number of intersects in the grid that fell within a root were counted, and the number of intersects where infection was present. This was repeated five times on different arrangements of the root sample.

Mycorrhizal colonisation was expressed as

$$\frac{\text{no. of intersects with colonisation}}{\text{no. of intersects}} \times 100$$

2.12 Plant Mineral Analysis

Mineral analysis of plants was carried out by Analytical Services Dept., SAC, Edinburgh. Leaves of experimental plants were collected at harvest and stored in polythene bags at 5°C until they could be processed. They were oven dried at 80°C and crushed using a pestle and mortar. Material was sent to SAC Edinburgh for analysis. Determinations were made of plant minerals by nitric acid digests of phosphorus, potassium, magnesium, calcium, sodium, sulphur, iron, boron, manganese, copper and zinc. Dissolution of oven dried and milled plant samples was brought about by the nitric acid digestion in a closed vessel. Pressure controlled microwave heating was used for the determination of metals, by plasma source optical emission spectrometry. The inductively coupled argon spectrometer was controlled by P.C. Results produced were transferred by file to the laboratory information management system LIMS. The limit of detection of minerals is given in Table 2.2 below.

Table 2.2 Limit of detection of minerals analysed by spectrometry

Element	Within batch standard deviation	Detection limit (%)
P	2.6 x 10-4	0.0012
K	7.864 x10-3	0.0357
Mg	2.66 x 10-4	0.00124
Ca	3.95 x 10-4	0.00184
Na	9.89 x 10-4	0.0046
S	4.62 x 10-4	0.00214
		(mg/kg)
Fe	1.985	9.23
B	0.1943	0.90
Mn	0.04825	0.22
Cu	0.2587	1.20
Zn	0.5075	2.36

CHAPTER 3

Comparison of drought tolerance of hybrid black poplar cv. Robusta when colonised by a range of arbuscular mycorrhizal species.

3.1 Introduction

Arbuscular mycorrhizal fungi in association with plant roots appear to have an influence on plant water relations. A number of direct and indirect mechanisms for this have been suggested. An overall decrease in root/shoot ratio has been shown in mycorrhizal plants relative to non-mycorrhizal plants in drought conditions, and interpreted as an improvement in drought tolerance (Busse and Ellis 1985). The root system of mycorrhizal plants has been shown to be more highly branched than non-mycorrhizal plants (Berta *et al.* 1990) which has an influence on the effectiveness of the root system in water uptake. It has been suggested that mycorrhizal colonisation alters the root resistance of the host plant (Safir *et al.* 1972) which has a direct effect on plant water uptake. The mycorrhizal effects on water relations of infected plants may also be a secondary consequence of the changes in phosphorus nutrition (Fitter 1988). The aim of this study was to examine the potential benefits that inoculation with arbuscular mycorrhizal fungi could provide in the response of trees to drought. One potential method of achieving this could be through mycorrhizal inoculation.

There is considerable interest in poplar species because their high growth rates make them useful tree species in small scale timber production, agroforestry, and shelter belts. Much work has been done on their response to drought (Edwards and Robertson 1975, Burgess *et al.* 1996). As a group poplars are relatively plastic in the range of types of fungi with which they can form associations, both ecto- and endomycorrhiza (Harley and Harley 1987). Relatively few workers use poplar in arbuscular mycorrhizal studies, with some exceptions (Tang and Chen 1995) who examined the benefit of mycorrhiza in disease resistance of poplar. Hooker *et al.* (1992) studied the change in root branching in hybrid black poplar var. Beaupre inoculated *with Scutellospora calospora, Glomus* species E3, or *Glomus caledonium.*

Although there have been many experiments with different crops, particularly high water requirement crops, using usually one or two mycorrhizal species, there

remains a need for a wider series of studies using tree species and comparing many species of mycorrhiza. A review of hybrid poplar varieties available in plant nurseries shows that 'Robusta', although less productive under ideal conditions than newer varieties such as 'Beaupre', is relatively popular because of its strong growth habit.

The aim of this work was to test the hypothesis that the response to imposed drought conditions of hybrid Black poplar cultivar Robusta varies as a result of colonisation with arbuscular mycorrhizal fungi. Two genera of mycorrhizal fungi were compared, *Glomus* which is a common group found in the U.K., and *Gigaspora*, a tropical group easily distinguished from other groups. From previous literature reviewed by Gupta (1991) various crop species have been shown to be positively or negatively affected by colonisation by mycorrhizal fungi, as discussed in Chapter 1. Resistance to water flow in the root system has been considered as a cause of variation in mycorrhizal response to drought (Safir *et al.* 1972, Levy and Krikun, 1980, Hardie and Leyton, 1981). An increased absorptive area in mycorrhizal plants has been shown (Fiscus 1977), and improved phosphorus nutrition also suggested as a mechanism for changes in drought response in mycorrhizal plants (Nelsen and Safir 1982).

This experiment was carried out to test the hypothesis that colonisation would improve the drought tolerance of hybrid black poplar cuttings. In this case a number of changes in their drought response would be seen. They would able to continue transpiring and photosynthesising longer during a drought period, and at a lower soil water potential. They would also have greater dry matter accumulation. A further hypothesis was that the particular host-fungal association would influence the response to drying.

3.2 Materials and Methods

3.2.1 Plant material

Hardwood cuttings of hybrid black poplar *Populus* x *canadensis* cv. Robusta (Tilhill Nurseries) with 4-5 buds were planted prepared soil in Magenta pots (Magenta Plant Cell Culture Vessel, Sigmaware) and kept under polythene until vigorous. They were kept in a glasshouse under 16 hours daylight from 06:00 to 22:00. Lighting was supplied by two 400W sodium lamps (Philips Son-T Agro), with a photon flux density of between 243 and 548 μmol m^{-2} s^{-1}. The temperature range was 8 to 17^{O}C.

3.2.2 Soil preparation

Thirty Magenta pots were sterilised with dilute 70% (v/v) ethanol. The weight of each Magenta pot was recorded to 0.1g accuracy, so this weight could be removed from the weight of soil. They were filled with Craibstone soil sieved to 3 mm. The soil had been previously autoclaved twice at 121^{O}C, 100 kPa for one hour. A moisture release curve is shown in Chapter 2 (Fig.2.1). The soil was packed to a bulk density of 0.82 g cm^{-3}. The volume of each Magenta pot was 380 cm^{3}. This meant that the mass of dry soil was 311.6 g, carefully measured on a balance to 0.1 g accuracy.

3.2.3 Inoculum

The soil was inoculated with one of four species of arbuscular mycorrhizal fungi. These were two *Glomus* species, *G.intraradices* and *G.mosseae*, and two *Gigaspora* species, *Gi.margarita* and *Gi.rosea*. Inoculum was obtained from pot culture using cucumber as the host species. Roots were harvested and examined microscopically for evidence of infection. Pieces of root with fungal structures present were selected. They were washed and chopped into 0.5 cm pieces. Where possible root pieces, hyphae and spores were all included in the selection. The maximum quantity available of each fungal species was gathered and divided into equal weights for inoculation. Variation in the quantity of mycorrhizal roots added, though not ideal, was imposed by the need to use uncontaminated inoculum, of which only small amounts could be obtained. The objective was to achieve rapid colonisation using the maximum quantity of clean inoculum available. This experiment was planned to be

long-term and it has been shown (Abbott and Robson 1985) that different species of mycorrhiza achieve different levels of colonisation in a host, despite the same quantity of inoculum. There were six replicates each of the following AMF species with fresh weights of inoculum as given in Table 3.1.

Table 3.1 Species and weight of inoculum per pot

Fungal species	INVAM code no.	Origin	Weight
Glomus intraradices	FL-208-4	Florida,U.S.	0.1g
Glomus mosseae	FL101A-1	Florida,U.S.	0.4g
Gigaspora margarita	WV205A-5*	W. Virginia, U.S.	1.0g
Gigaspora rosea	BR155B-1	Brazil	0.6g
Uncolonised roots			0.5g

3.2.4 Method

In this system where water use is determined gravimetrically, evaporation must either be accounted for, or prevented. If the pot surface is sealed, the soil atmosphere will change. Because of the duration of this experiment, there was some concern over potential changes in the soil atmosphere if the pots were sealed for a long period of time. For this reason, in the following experiments, the pots were not sealed, but evaporation was reduced by covering the soil with 20 g 2 mm diameter gravel. Water use of mycorrhizal plants was compared relative to non-mycorrhizal plants. The pots were weighed daily at 12:00 to calculate their soil moisture content. A series of drying cycles was imposed on all the pots. The water use (ml) and the dry matter accumulation (g) of the plants was measured over one growing season from April to November, to examine the influence of these mycorrhizal species on the drought response of these plants. Plants were subjected to drying cycles of 5 days duration, after which they were rewatered. Between each drying cycle was a resting period of one week. During "resting" periods, plants were kept well watered, according to requirement, but still maintaining the same water quantity supplied to all plants.

Half strength Hoaglands solution (Hoagland and Arnon 1938) was applied in the watering regime approximately monthly. The recipe for Hoaglands solution is given in Appendix 2.

After harvest the fresh and dry weights of the roots and shoots were measured. The length of the root system was found using image analysis of root samples, described in Chapter 2. The leaf area was also found using image analysis. The mycorrhizal colonisation was assessed using the method given in Chapter 2. The nutrient status of the non-woody parts of the shoot was measured by the Analytical Services Dept., SAC, Edinburgh, described in section 2.11.

3.3 Results

3.3.1 Mycorrhizal colonisation

A sample was taken from the root system of each plant and used to assess the mycorrhizal status of the roots. The sample was weighed, then cleared and stained using the procedure described in Chapter 2 section 2.10. The proportion of mycorrhizal colonisation is given as the percentage of the total root length in which evidence of mycorrhizal structures, either arbuscles, vesicles or internal hyphae, were found. Data for mycorrhizal colonisation is given in Table 3.2 below.

Table 3.2 Percentage mycorrhizal colonisation of plants at harvest

	Mycorrhizal species				
Replicate	Control	*G.intraradices*	*G.mosseae*	*Gi.rosea*	*Gi.margarita*
1	0%	33%	11%	0%	29%
2	0%	36%	22%	0%	54%
3	0%	0%	28%	17%	0%
4	0%	0%	37%	0%	0%
5	0%	24%	25%	11%	22%
6	0%	0%	15%	-	17%
Mean of colonised plants	0%	31%	23%	14%	30%

Some replicate plants failed to become colonised by AMF. Data from these plants was not used in further calculations.

3.3.2 Plant characteristics

The growth characteristics of the plants are shown in Table 3.3. The leaf area of the plants at harvest was measured using a Quantimet 600 image analysis system. The root length of each plant colonised by different mycorrhizal species was derived from samples taken randomly from the total root system. The samples and the original root system were oven dried. The root length of the sample was measured using a Quantimet 600 image analysis system. The total length of the root system was then calculated on the basis of the length/weight relationship of the sample. This is detailed in Chapter 2 section 2.9.

One way analysis of variance was performed on each characteristic to determine differences between species. There was no significant difference between species except in shoot weight. In general shoot weight was reduced by mycorrhizal colonisation. Those colonised by *Gi.rosea* showed the lowest shoot weight. However plants associated with *G.intraradices* showed an increase in weight.

Root weight was similar in all plants, but specific root length was increased in *Gigaspora* species, particularly *Gi.rosea,* which showed almost 15 m greater length than the non-mycorrhizal control. In general the root/shoot ratio was increased in mycorrhizal plants, except for those associated with *G.intraradices*.

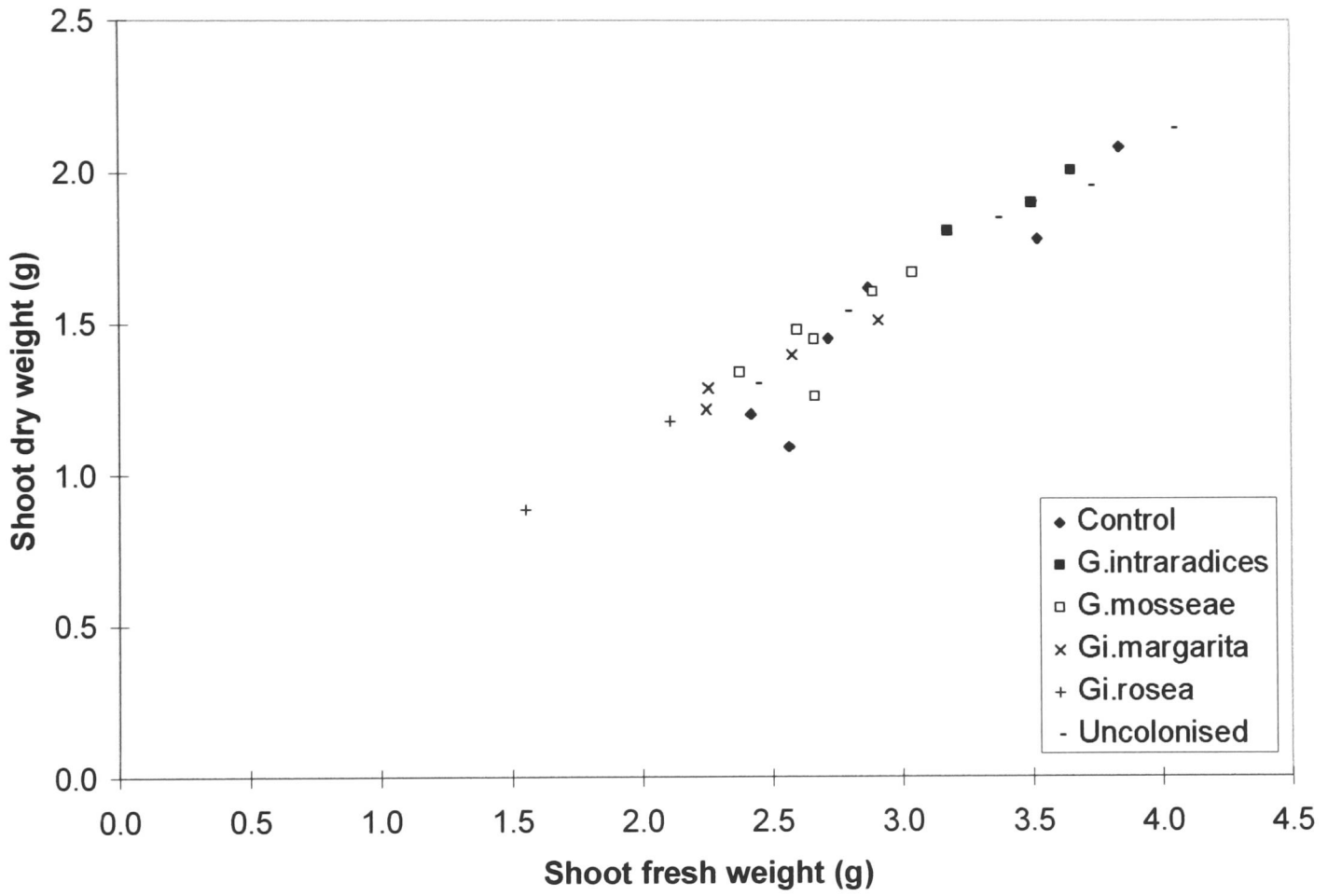
Table 3.3 Summary of growth characteristics in plants inoculated with different mycorrhizal species
Mean

Species	Leaf area (cm^2)	Fresh shoots (g)	Dried shoots (g)	Fresh roots (g)	Dried roots (g)	Specific root length(m/g)	Root length (m)	Root/ shoot ratio
control	92.7	3.0	1.5	17.3	2.3	188.6	446.2	1.5
G.intraradices	101.1	3.4	1.9	14.5	2.7	170.4	447.4	1.5
G.mosseae	117.0	2.7	1.5	20.1	2.6	186.2	495.6	1.8
Gi.margarita	126.8	2.5	1.4	16.7	2.3	213.1	488.9	1.7
Gi.rosea	119.0	1.8	1.0	17.8	2.4	244.6	592.5	2.4
uncolonised	123.6	3.3	1.8	22.8	2.9	205.9	602.5	1.7
Standard error								
control	27.3	0.2	0.2	4.1	0.4	18.7	82.6	0.3
G.intraradices	24.4	0.1	0.1	3.2	0.7	18.4	84.1	0.4
G.mosseae	27.6	0.1	0.1	2.1	0.2	18.7	68.6	0.1
Gi.margarita	24.7	0.2	0.1	1.6	0.4	24.0	108.3	0.3
Gi.rosea	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
uncolonised	12.4	0.2	0.1	3.2	0.4	14.5	72.8	0.2
	NS	**	*	NS	NS	NS	NS	NS

Those plants which failed to become colonised by fungi are also shown above. Their root system weight and length was greater than any other group. For all other characteristics, they showed values within the range shown by control and mycorrhizal plants.

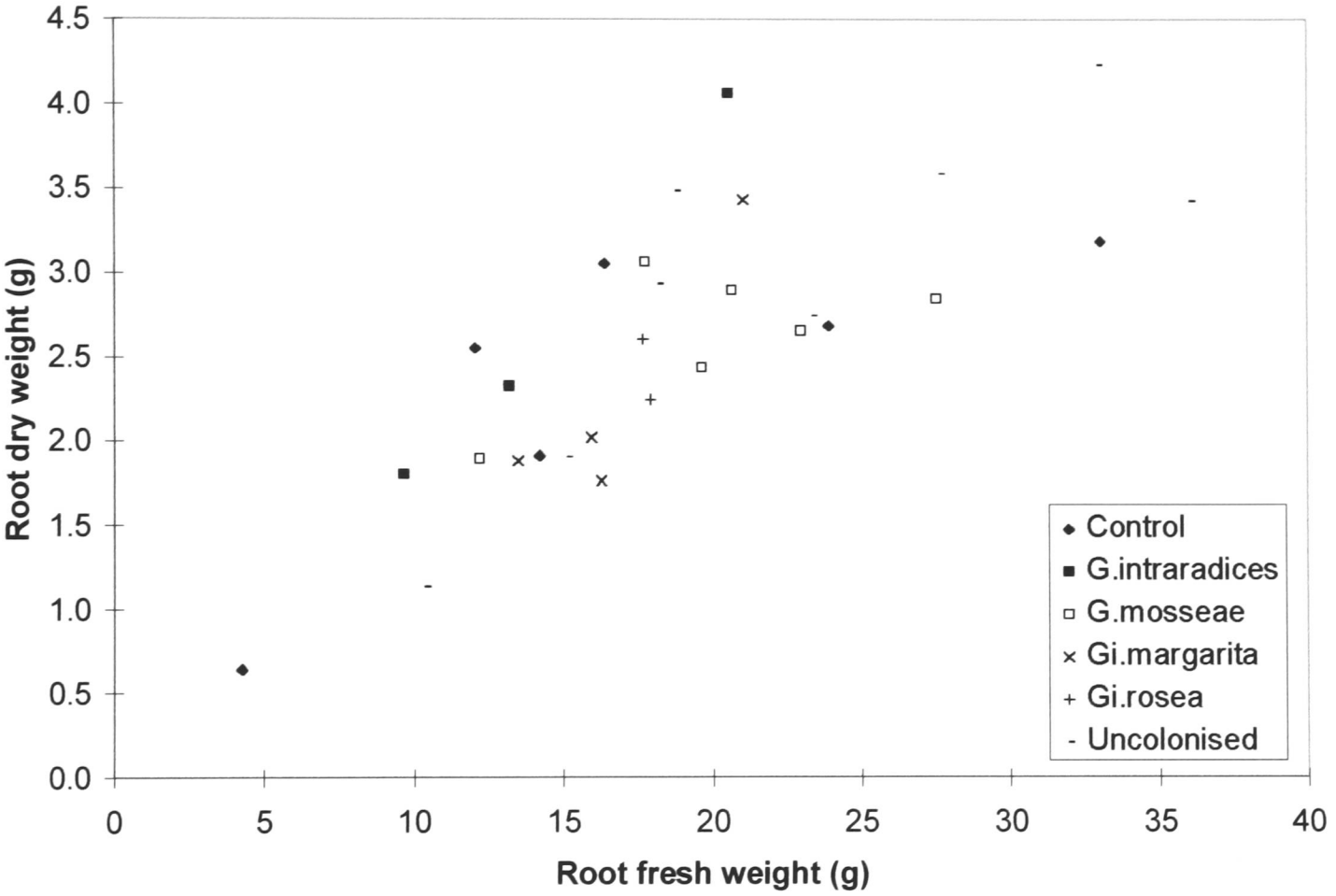
A comparison of the harvested samples was carried to ensure their validity. Fresh and dry weights of the root system and shoots were compared. Shoot fresh and dry weights (Fig.3.1) showed a clear linear relationship. Four samples deviated slightly from this relationship. These were three control samples and one mycorrhizal *Glomus mosseae* sample, which showed lower dry weights relative to their fresh weight than the others. There may have been an error in weighing these samples when fresh, but in general sample weighing showed consistency.

Fig.3.1 Shoot fresh weight and dry weight of samples at harvest



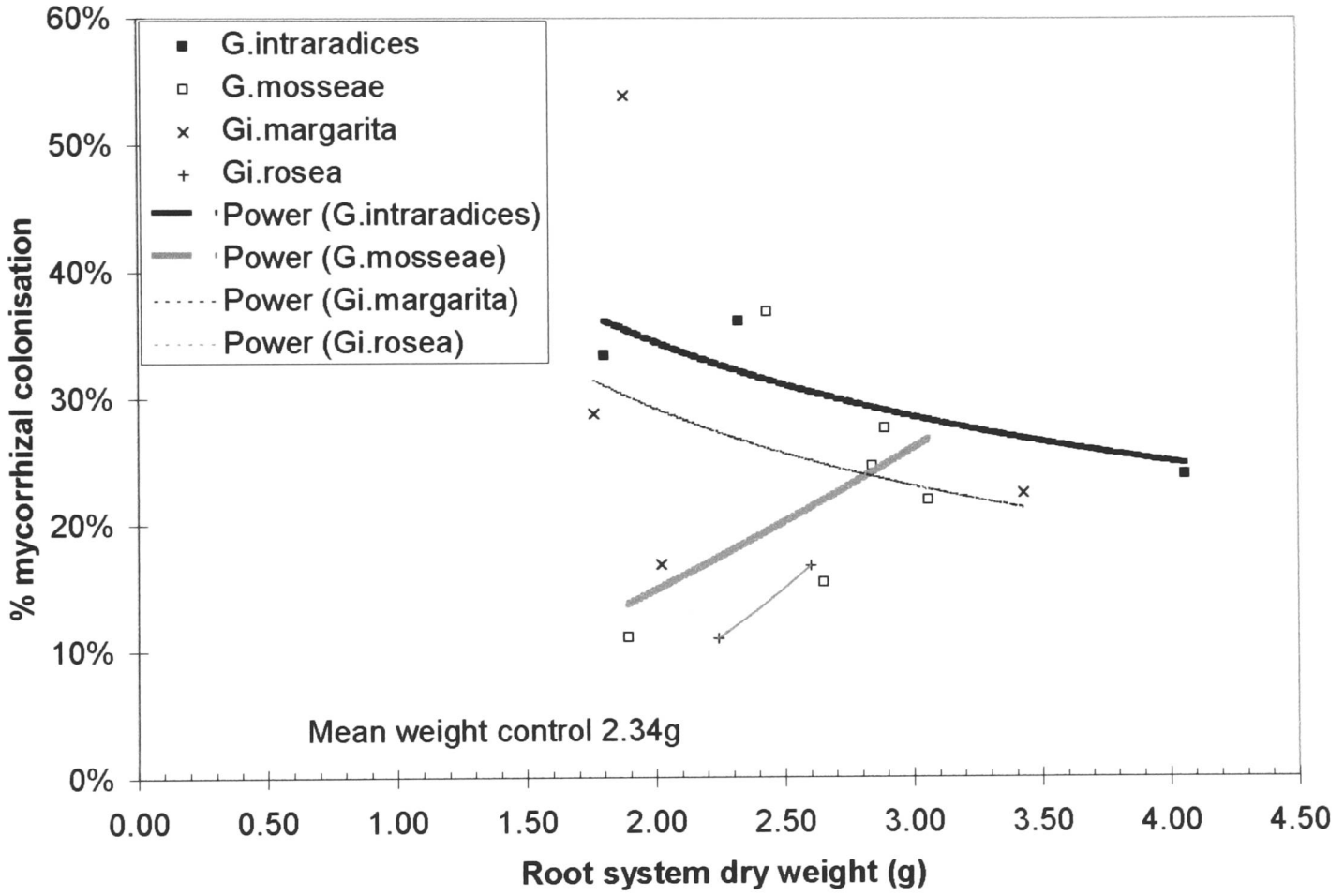
There was greater variation in the relationship between root system fresh and dry weights (Fig.3.2). Samples appeared to fit two linear relationships. The relationship between weights of samples of *Glomus intraradices* and *G. mosseae* mycorrhizal plants in particular appeared to diverge. This may have been due to differences in the root system architecture of these plants. Those with greater suberization were likely to show higher dry weights relative to their fresh weight. Alternatively these results may have been due to greater drying of some samples as their fresh weights were being measured.

Fig.3.2 Root fresh weight and dry weight of samples at harvest



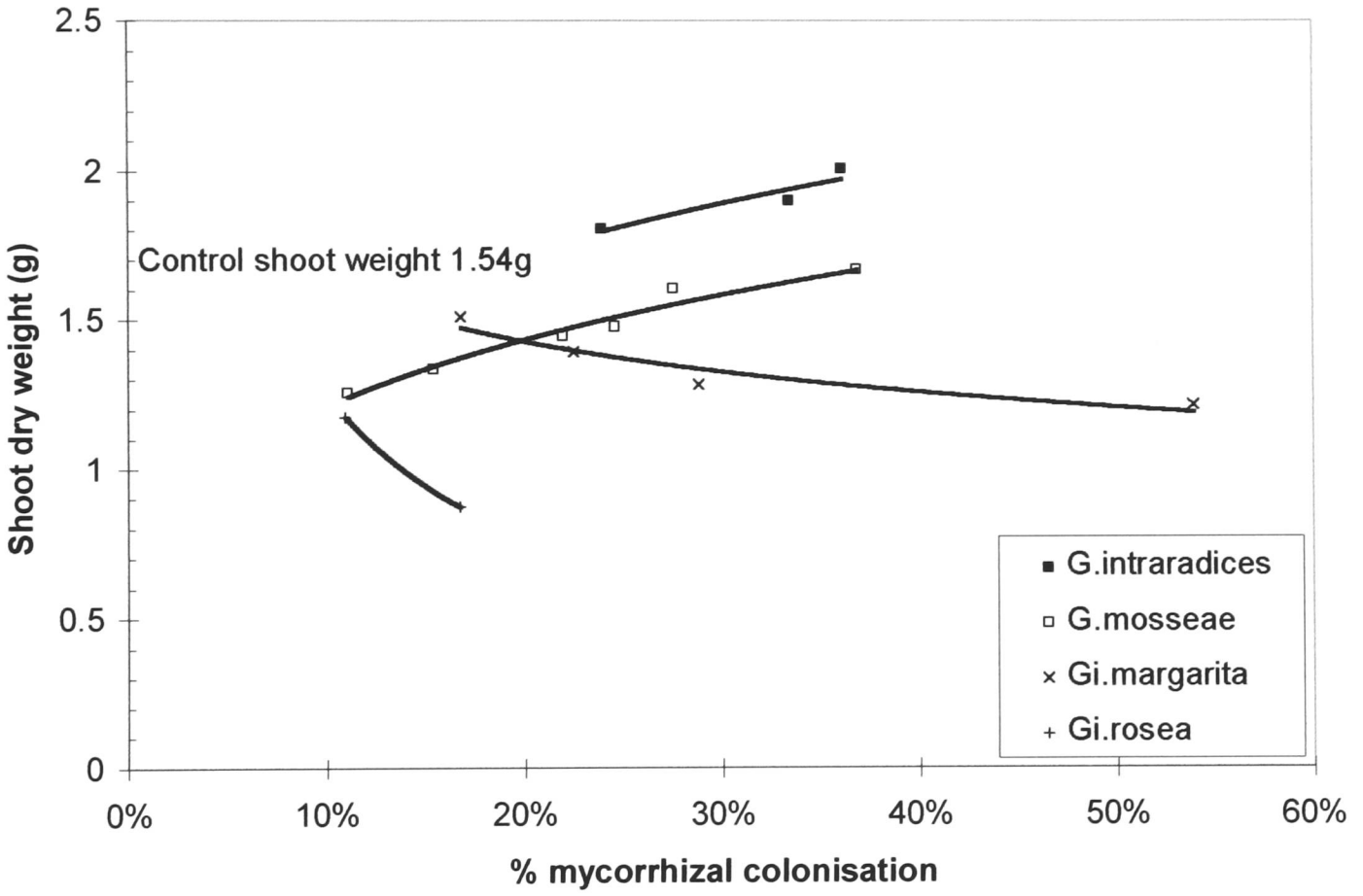
The proportion of root colonisation by mycorrhiza was plotted against the shoot and root weight. The dry root weight of plants colonised by *G.intraradices* and *Gi.margarita* showed a decrease with increased mycorrhizal colonisation (Fig.3.3). Where plants were colonised by *G.mosseae* or *Gi.rosea*, there was an increase in dry root weight with increasing mycorrhizal colonisation. The control dry root weight was 2.34 g. Multiple linear regression was carried out on the relationship between root dry weight, mycorrhizal species and percentage mycorrhizal colonisation. No significant difference between samples was found.

Fig.3.3 Mycorrhizal colonisation compared with root system dry weight



Shoot dry weight appeared to increase with increasing mycorrhizal colonisation in *Glomus* species, but to decrease in *Gigaspora* species (Fig.3.4). The control value lay midway between the mycorrhizal species at 1.54 g dry weight. Multiple linear regression was carried out on the shoot weight, the mycorrhizal species and the percentage mycorrhizal colonisation. This difference in shoot dry weight between mycorrhizal species was significant at 1%. The relationships between shoot dry weight and mycorrhizal colonisation were not significant.

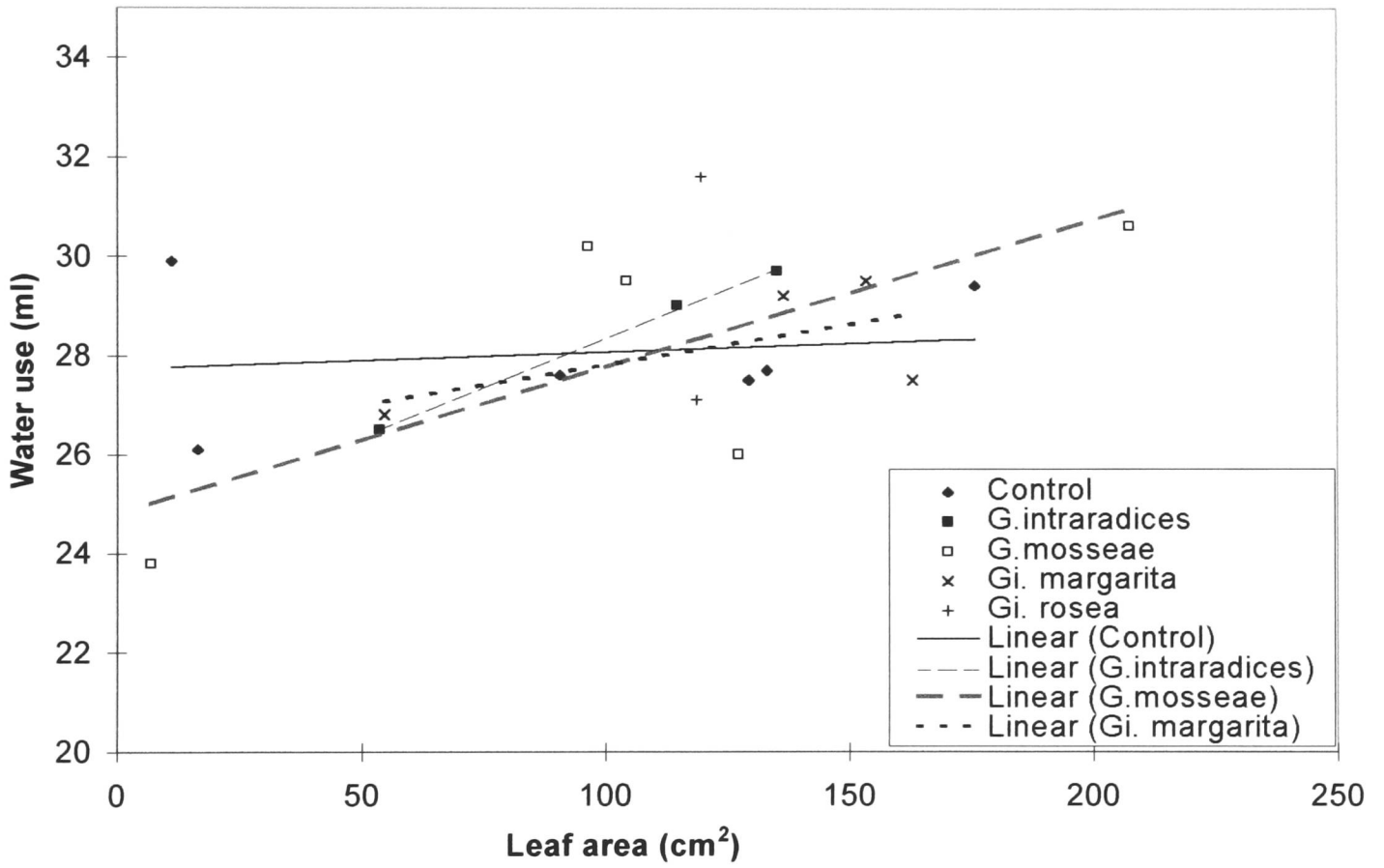
Fig.3.4 Shoot dry weight compared with mycorrhizal colonisation



3.3.3 Water use and leaf area

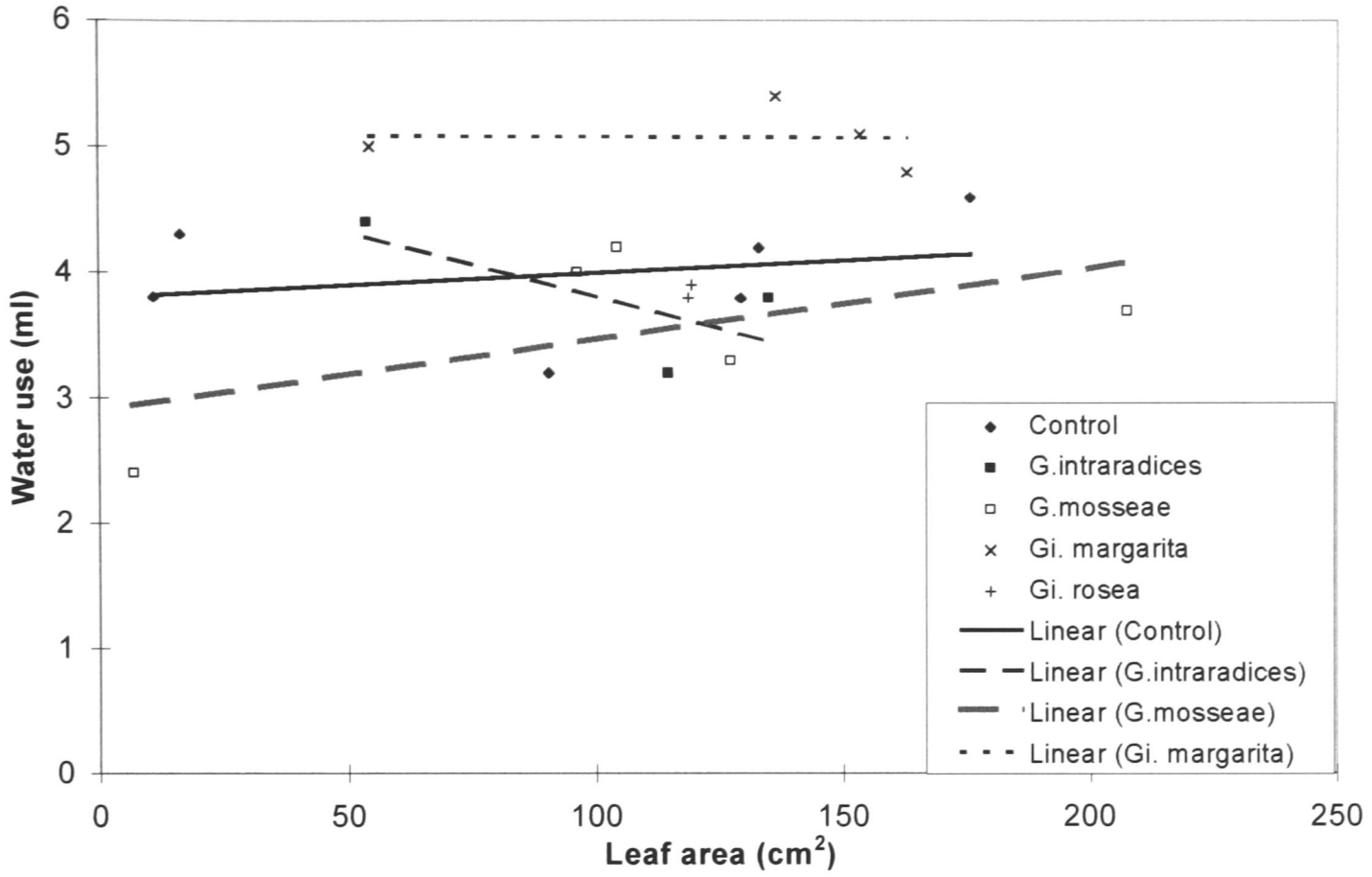
There was consistently increased leaf area in mycorrhizal plants (Table 3.3). The leaf areas and daily water use for plants inoculated with the different mycorrhizal species are plotted for maximum transpiration at the beginning of a drying cycle and minimum transpiration at the end (Figs.3.5,3.6). This shows the dependence of transpiration on leaf area at maximum and minimum transpiration. Due to their increased leaf area mycorrhizal plants show increased rates of water use despite their generally lower gas exchange.

Fig.3.5 Daily water use at maximum transpiration compared to leaf area for plants inoculated with one of four AMF species.
Trend lines are fitted by linear regression.



At the time of maximum transpiration there was a clear significant ($p=0.02$) relationship between leaf area and water use. There was no indication of any differing response between plants colonised by different mycorrhizal species ($p=0.9$). Nor was there any difference between mycorrhizal plants and control non-mycorrhizal plants.

Fig.3.6 Daily water use at minimum transpiration compared to leaf area for plants inoculated with one of four AMF species.
Trend lines are fitted by linear regression



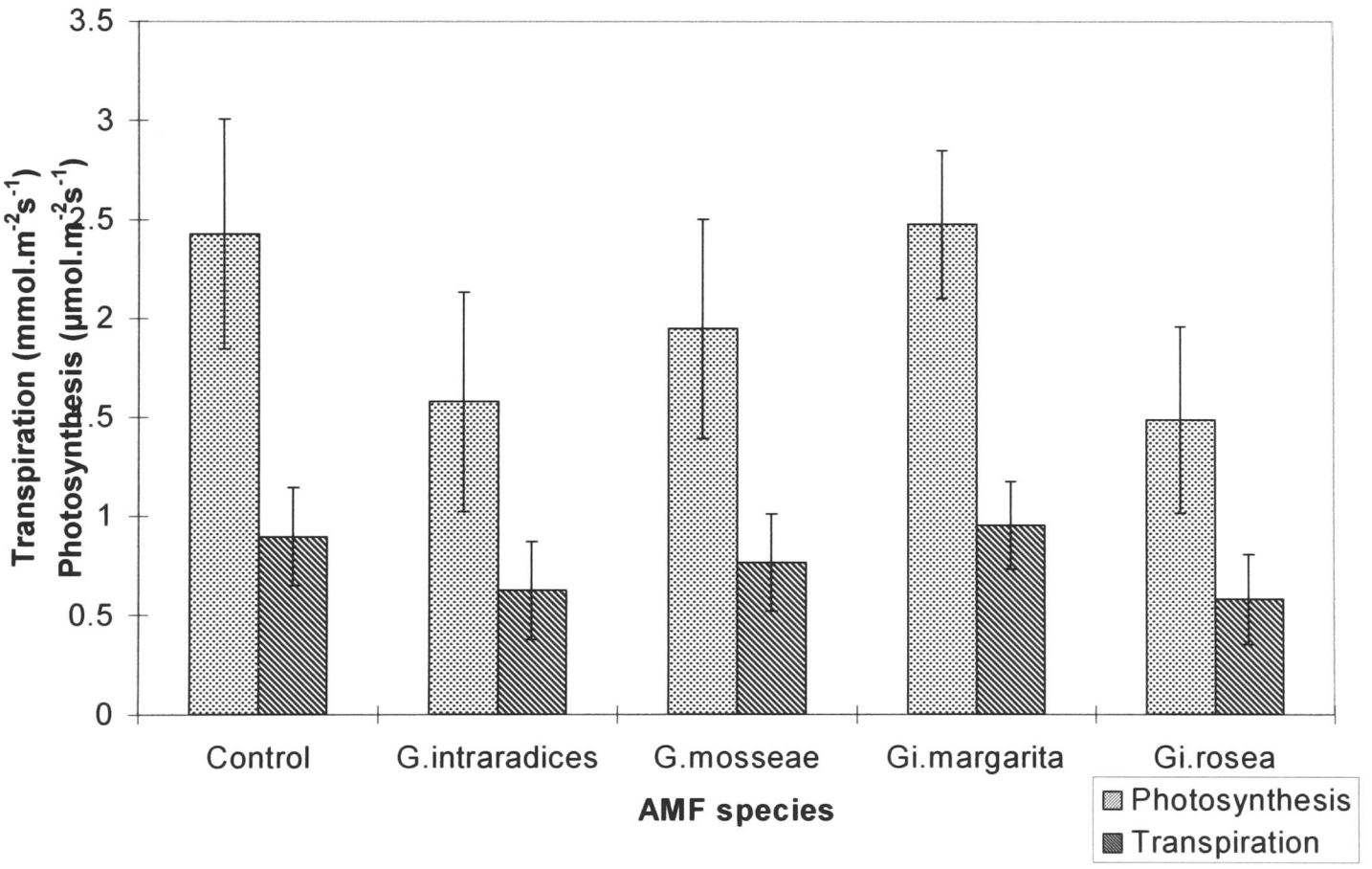
At the time of minimum transpiration, there was no significant relationship between leaf area and water use ($p=0.5$). For the majority of species water use was little affected by a change in leaf area. This is confirmed by two-way analysis of variance on the data which indicated no significant relationship between either leaf area and water use or species and water use. The values for *Glomus intraradices* differ in that they show decreased water use with increasing leaf area.

3.3.4 Shoot responses

The rate of transpiration and photosynthesis of the plants, and the stomatal conductances of the leaves were measured for each treatment throughout the experiment. A sample of 12 days of measurements which represented well the general patterns of shoot responses of the plants is shown in Figs.3.7 and 3.8. Transpiration and photosynthesis are shown in Fig.3.7. There were similar responses in these two processes. The control plants and those inoculated with *Gi. margarita* showed the highest rates of these processes. Those inoculated with *G.intraradices* and *Gi.rosea* showed the lowest rates. One way analysis of variance between treatments showed that there was no significant difference in transpiration nor photosynthesis between mycorrhizal species or the control.

Fig.3.7 Transpiration and photosynthesis in plant inoculated with different mycorrhizal fungi

Standard error bars are shown. Mean for 12 days

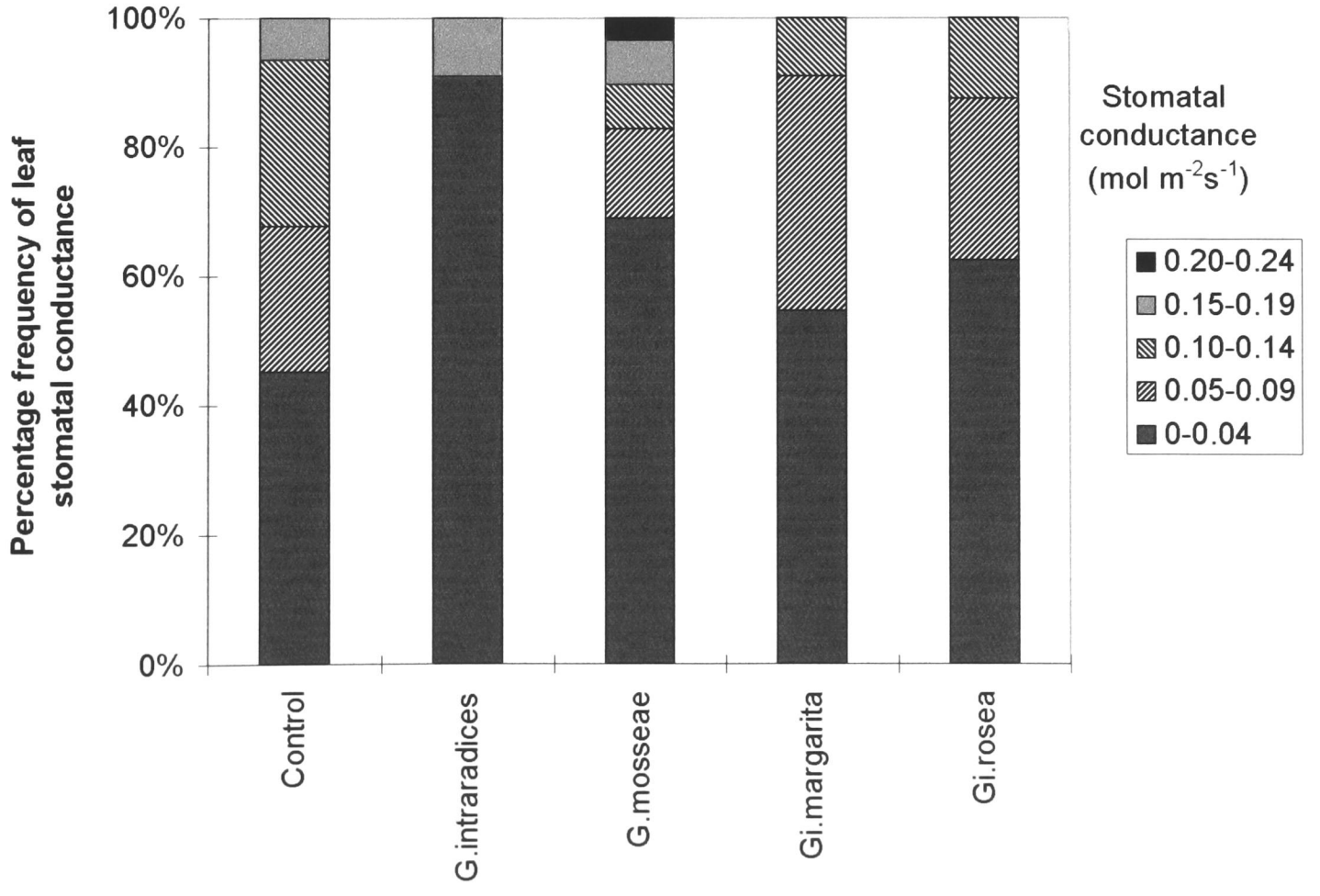


The stomatal conductance of the plant leaves was also measured throughout the experiment. The mean stomatal conductance for each treatment over the same sampled days, showed the same responses as in Fig. 3.7. Generally stomatal conductance was higher in the uninoculated plants. Those inoculated with *Gi.margarita* also had high stomatal conductances, whereas those colonised by

G.intraradices or *Gi.rosea* showed a large reduction in stomatal conductance relative to control plants. In order to consider the shoot responses more fully, the range in stomatal conductances which gave rise to these responses was examined. The stomatal conductance varied widely between plants and over the course of time. The results for each mycorrhizal treatment, including the control, were collected. They were arranged in order of increasing stomatal conductance for each treatment. They were classified into a range of conductances. The frequency of each group where noted and converted to percentage frequency of the whole sample. *G. intraradices* and *Gi. rosea*, with low mean shoot responses, showed a high proportion of stomatal conductance values in the low ranges. Control plants and *Gi. margarita* showed a higher proportion of high value stomatal conductances. These plants also showed the highest mean stomatal conductance, transpiration and photosynthetic rates. The plants associated with *G. mosseae* showed the widest range of stomatal conductance values, but a relatively high proportion of low values. Kolmogorov-Smirnov analysis was carried out on these results. Differences in stomatal conductance were significant at 1%.

Fig.3.8 Comparison of stomatal conductance in plants inoculated with different mycorrhizal fungi

Mean for 12 sampling days



3.3.5 Plant nutrient status

The nutrient contents of the non-woody shoots of each plant are presented in Table 3.4. Differences in nutrient concentration were analysed for each nutrient by one-way analysis of variance. The only significant differences in nutrient content between the different AMF treatments were potassium and magnesium. Plants infected with *Gi.rosea* were significantly lower in magnesium concentration. Plants inoculated with *Gigaspora* species were higher in potassium concentration than the uninoculated plants. In particular there was no significant difference in phosphorus content (p=0.07), although plants inoculated with *G.intraradices* had almost twice the phosphorus concentration of other plants. However there were some individual differences noted. In *Gi.rosea* infected plants, the iron content was 3% higher than the other treatments. All mycorrhizal plants showed reduced manganese concentration, although the difference was not significant. The plants inoculated with *Glomus* species showed the lowest concentration of this nutrient. Boron, calcium and zinc concentrations were also reduced in comparison with the control. The concentration of nitrogen in general was rather low. It would have been expected to be above 2% (Smith 1966), but was generally about 1% dry matter.

Table 3.4 Mean nutrient concentration of mycorrhizal and non-mycorrhizal plants

Species	Phosphorus %DM	Nitrogen %DM	Potassium %DM	Magnesium %DM	Calcium %DM	Sodium %DM	Sulphur %DM
control	0.19	1.15	1.18	0.33	2.36	0.03	0.38
G.intraradices	0.31	0.68	1.23	0.37	2.05	0.03	0.41
G.mosseae	0.16	0.88	1.16	0.33	1.84	0.03	0.34
Gi.margarita	0.19	1.08	1.63	0.32	1.97	0.02	0.34
Gi.rosea	0.16	1.08	1.97	0.24	1.87	0.01	0.34
Significant difference	NS	NS	*	**	NS	NS	NS

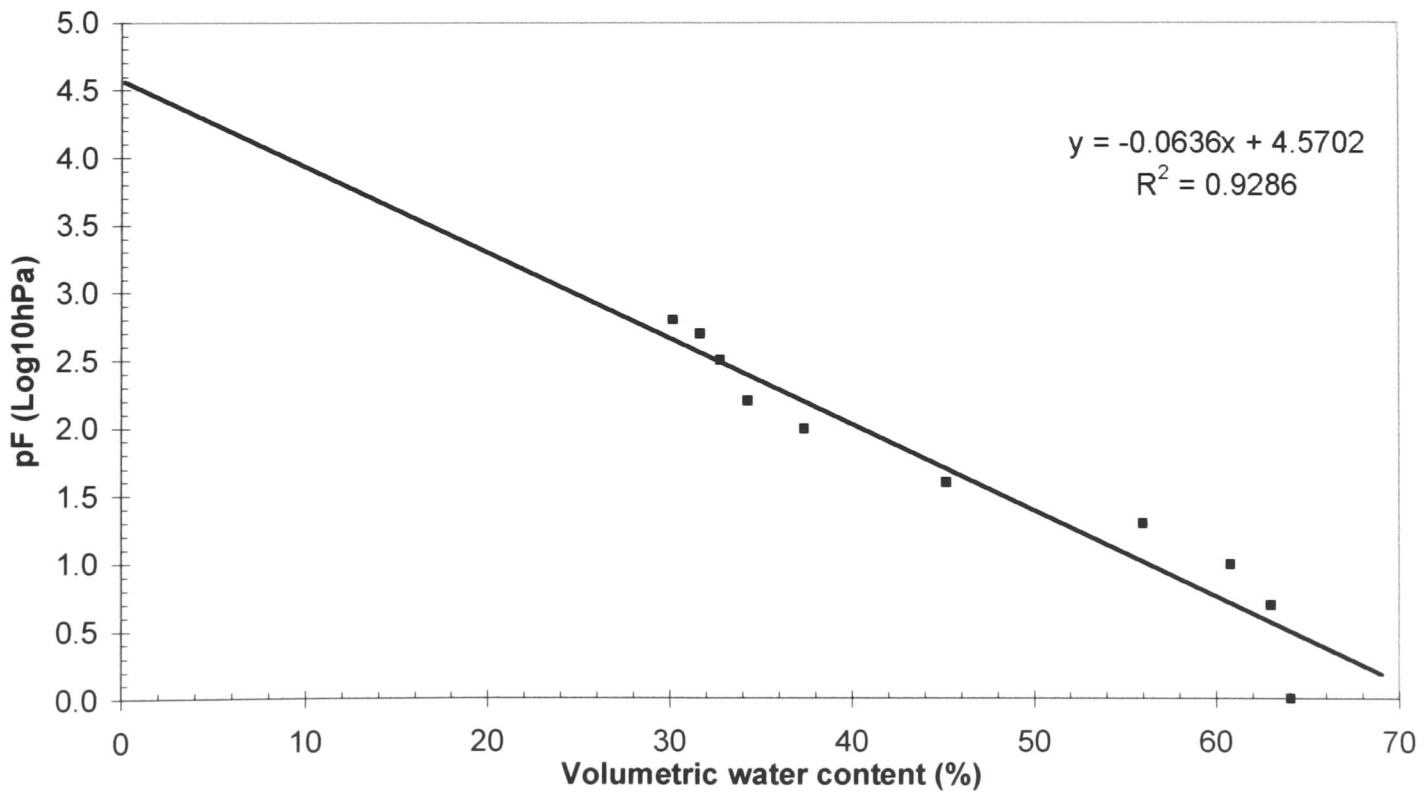
	Iron mg/kgDM	Copper mg/kgDM	Manganese mg/kgDM	Boron mg/kgDM	Zinc mg/kgDM
control	71	3.6	719	77	140
G.intraradices	61	2.7	508	52	140
G.mosseae	45	3.8	453	39	75
Gi.margarita	67	3.2	388	59	87
Gi.rosea	131	3.4	363	51	116
Significant difference	NS	NS	NS	NS	NS

3.3.6 Comparison of water use between mycorrhizal and non-mycorrhizal plants

Two-way analysis of variance was carried out on the variation in water use between mycorrhizal treatments. There was no significant difference in daily weight change i.e. water use between non-mycorrhizal and mycorrhizal plants, ($p=0.2$), and no interaction with time. The non-mycorrhizal plants initially had greatest water use, but had reduced water use relative to non-mycorrhizal plants after 3 months of monitoring.

The response in water use at varying soil water potentials, at four instants during the experiment, was examined. The soil water potential was calculated by using the weight of water in the soil, to estimate the volumetric water content. The moisture release curve for Craibstone soil (Fig.3.9) was used to convert the volumetric water content to soil water potential value. The soil water release curve for Craibstone soil and its use to calculate soil water potentials from soil water content was detailed in Chapter 2 section 2.2.

Fig.3.9 Relationship between volumetric soil water content and soil water potential

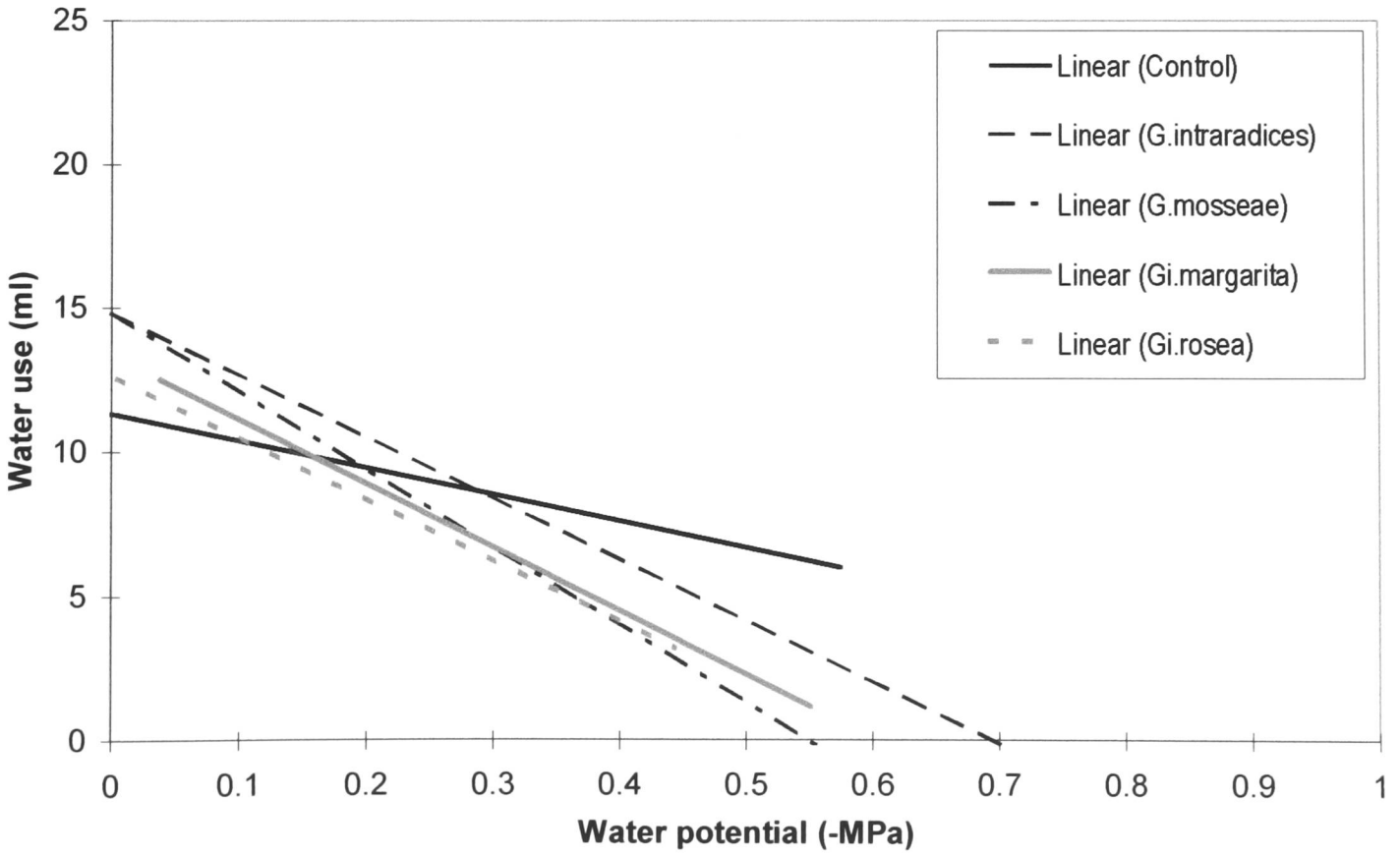


Analysis of variance was performed on the data for daily water use and on the calculated soil water potentials at various dates during the course of the study. In general there was no significant difference between mycorrhizal and non-mycorrhizal plants in their response to soil water potential. No significant difference was found between mycorrhizal treatments, ($p=0.3$). The maximum water potential found was -0.01 MPa and the minimum -3 MPa. The calculated soil water potential and the daily water use, for each mycorrhizal species, for four periods during the study, were plotted as scatter graphs (Figs.3.10-3.13). Linear regressions were calculated for these relationships. The probabilities of significance varied greatly. These are shown in Table 3.5.

It was noticed that the various treatments gave very different responses with time for similar starting soil water potentials. The first period was in August, the second in November the third in May and the fourth in October. During the first period, in the first weeks after inoculation, water potentials of 0 to -0.7 MPa were experienced by the plants (Fig.3.10). Mycorrhizal plants appeared to have a higher rate of water use at saturation, than the control plants. The mycorrhizal plants also appeared to be more sensitive to drying than the control plants, in that their water use decreased more rapidly with decreasing water potential. No obvious difference was seen between mycorrhizal treatments. The relationship between water use and soil water potential was significant, but not between mycorrhizal treatments, or the control. Although the differences in the response of water use to decreasing water potential between species were not significant, against the conventional standard, comparison of *G.mosseae* and the control (p=0.07), and between *G. intraradices* and the control (p=0.07), were close to conventional significance and may be taken as indicating a possible difference.

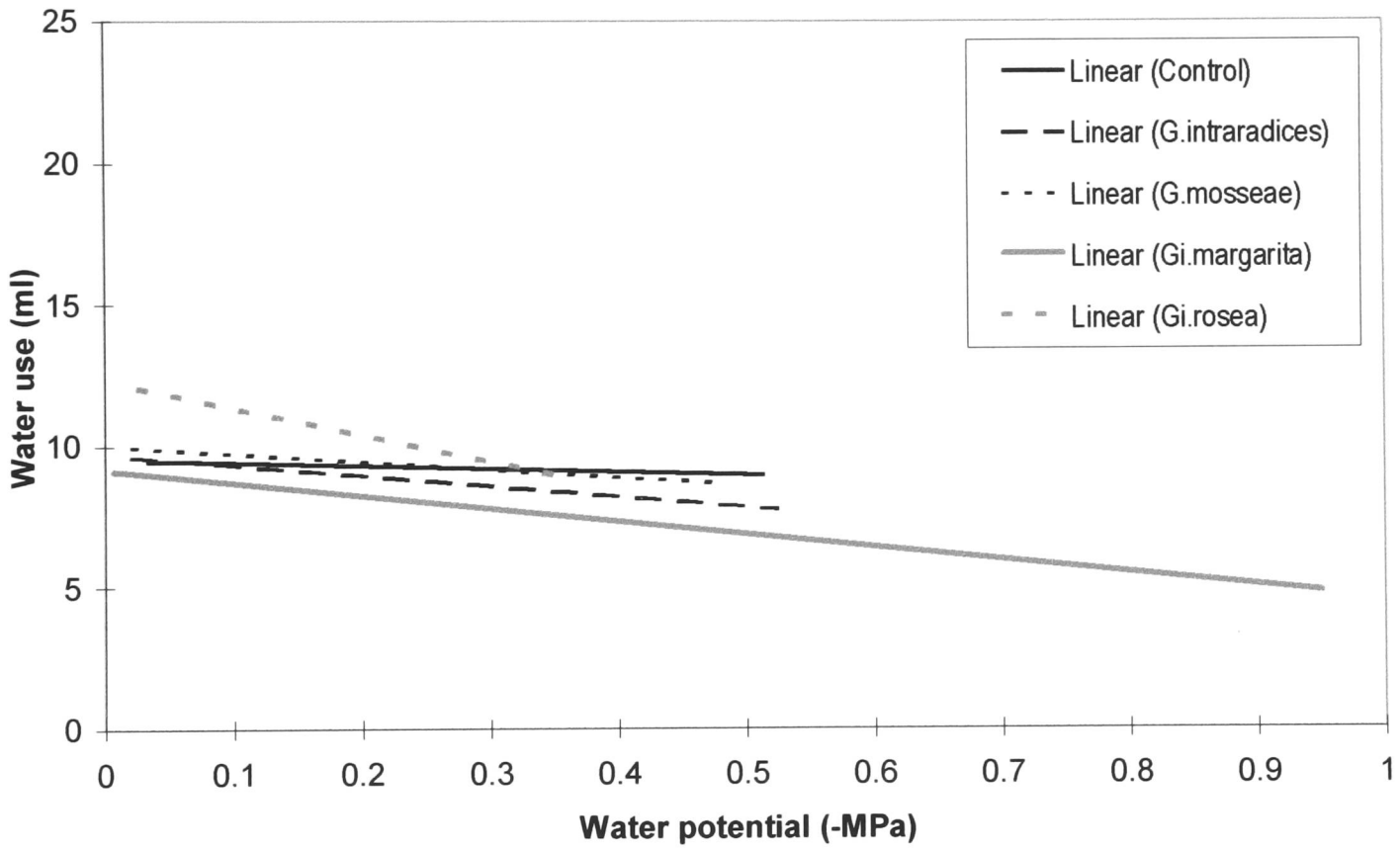
Fig.3.10 Plant water use compared with soil water potential when inoculated with different mycorrhizal species - period 1: one month after inoculation

Trend lines are fitted by linear regression



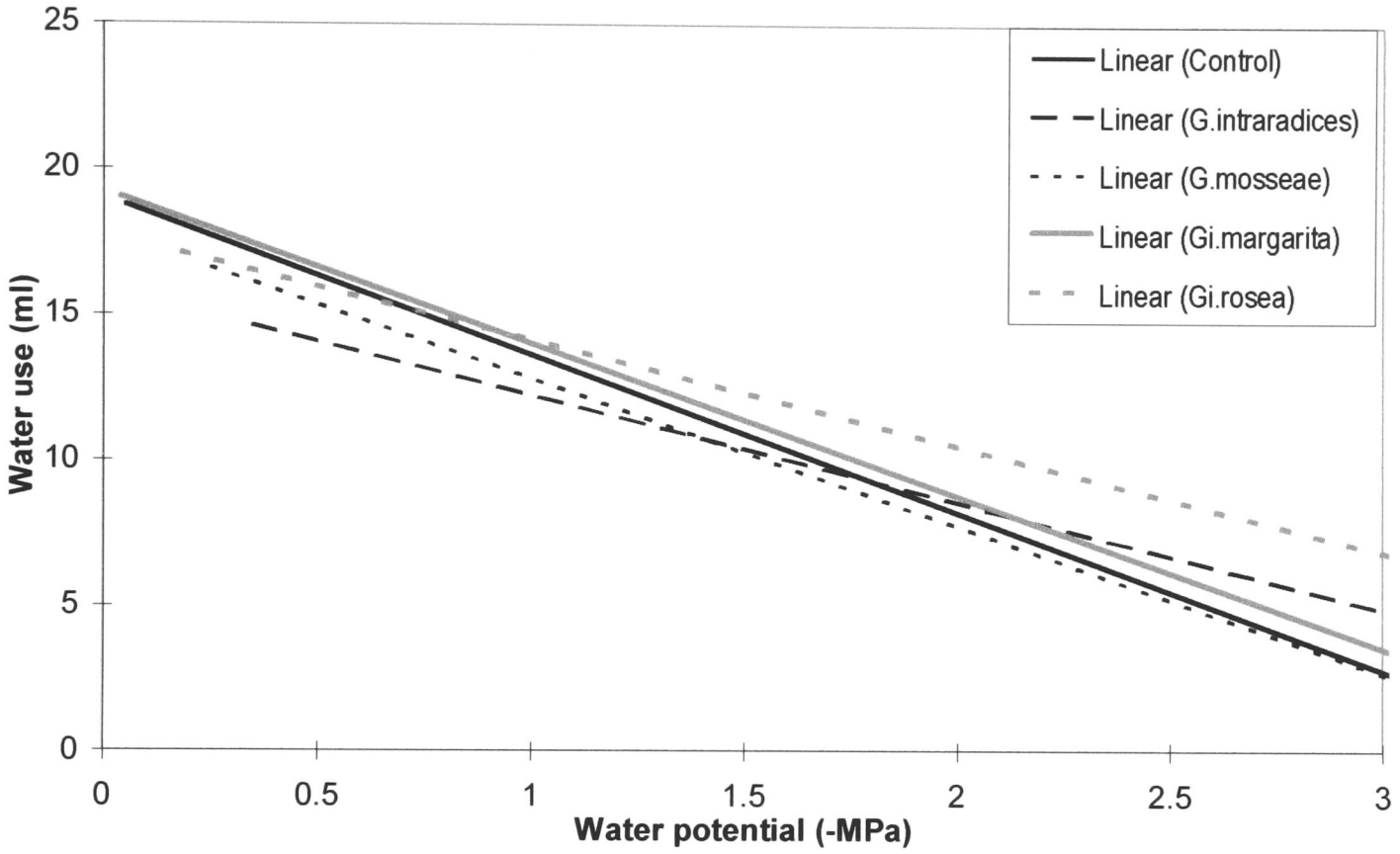
During the second period, three months after inoculation, the minimum water potential experienced was almost -1 MPa (Fig.3.11). Water use no longer showed a significant response to water potential, although there was still a general decrease in water use with water potential. Only plants colonised by *Gi.rosea* showed a greater sensitivity to decreasing soil water potential. However this difference was not significant (p=0.2). Since this period occurred in the autumn, radiation was lower. As a consequence drying of the soil occurred over a longer period.

Fig.3.11 Plant water use compared with soil water potential when inoculated with different mycorrhizal species - period 2: 3 months after inoculation
Trend lines are fitted by linear regression



During the third period, 10 months after inoculation, much lower water potentials of -3 MPa were experienced (Fig.3.12). The decrease in water use with soil water potential was significant. Plants colonised with *Gi.rosea* or *G.intraradices* appeared to be less sensitive to water potential, but not significantly different.

Fig.3.12 Plant water use compared with soil water potential when inoculated with different mycorrhizal species - period 3: 10 months after inoculation
Trend lines are fitted by linear regression



During the fourth period, 14 months after inoculation, the minimum soil water potential was less severe at approximately -0.8 MPa for most plants (Fig.3.13). There was no significant relationship between water use and soil water potential, nor between mycorrhizal treatments, although those infected with *G.mosseae*, in this case, appeared to show reduced sensitivity to decreasing soil water potential.

Fig.3.13 Plant water use compared with soil water potential when inoculated with different mycorrhizal species - period 4, 14 months after inoculation
Trend lines are fitted by linear regression

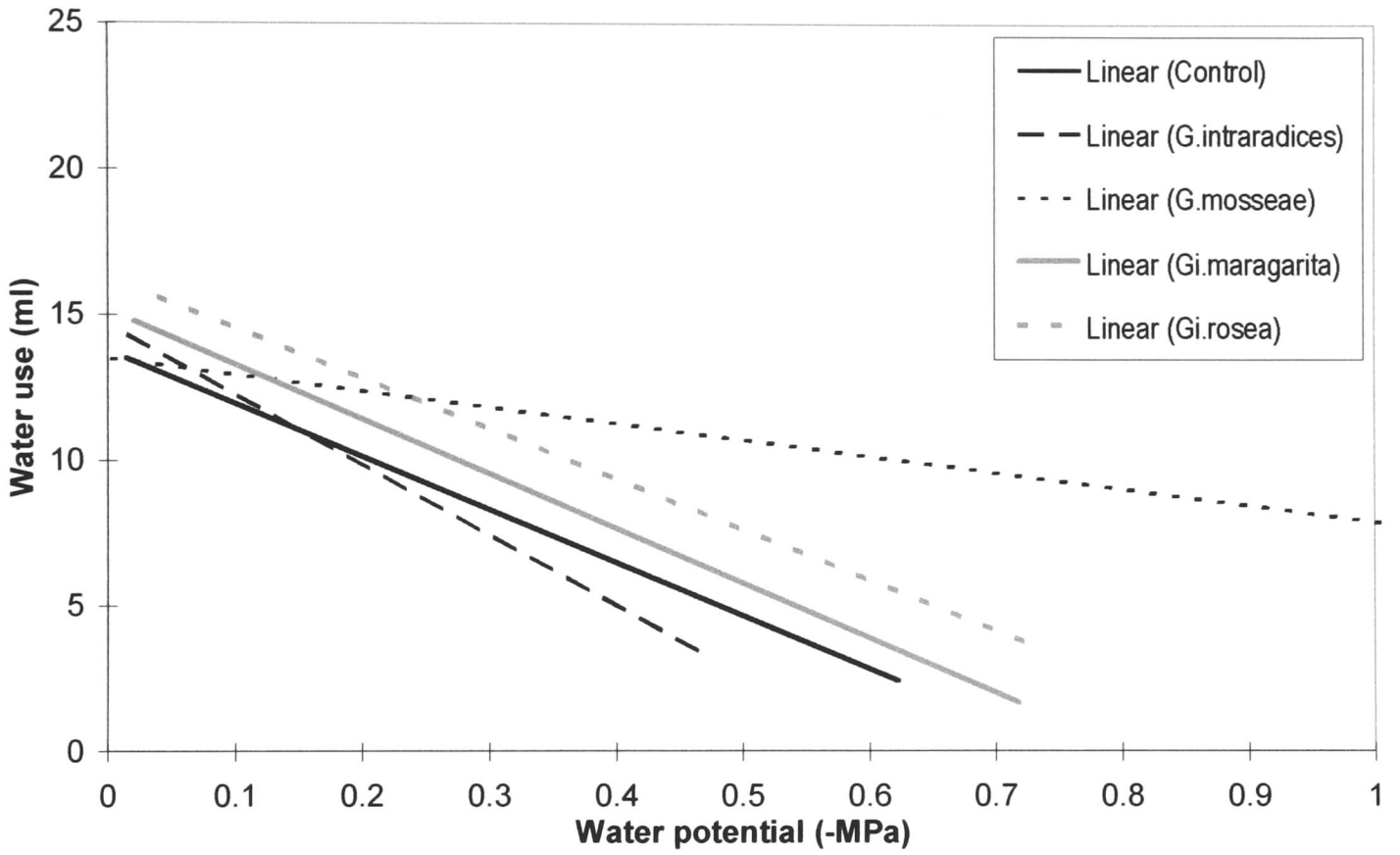


Table 3.5 gives a summary of the probability of a relationship between water use and soil water potential, for each mycorrhizal association.

Table 3.5 Summary of Analysis of Variance of slope of Water use versus Soil Water Potential regression lines.

	Period			
Species	1	2	3	4
Control	NS	NS	***	(NS)
G.intraradices	**	NS	**	NS
G.mosseae	***	NS	***	NS
Gi.margarita	**	NS	***	(NS)
Gi.rosea	**	NS	**	NS

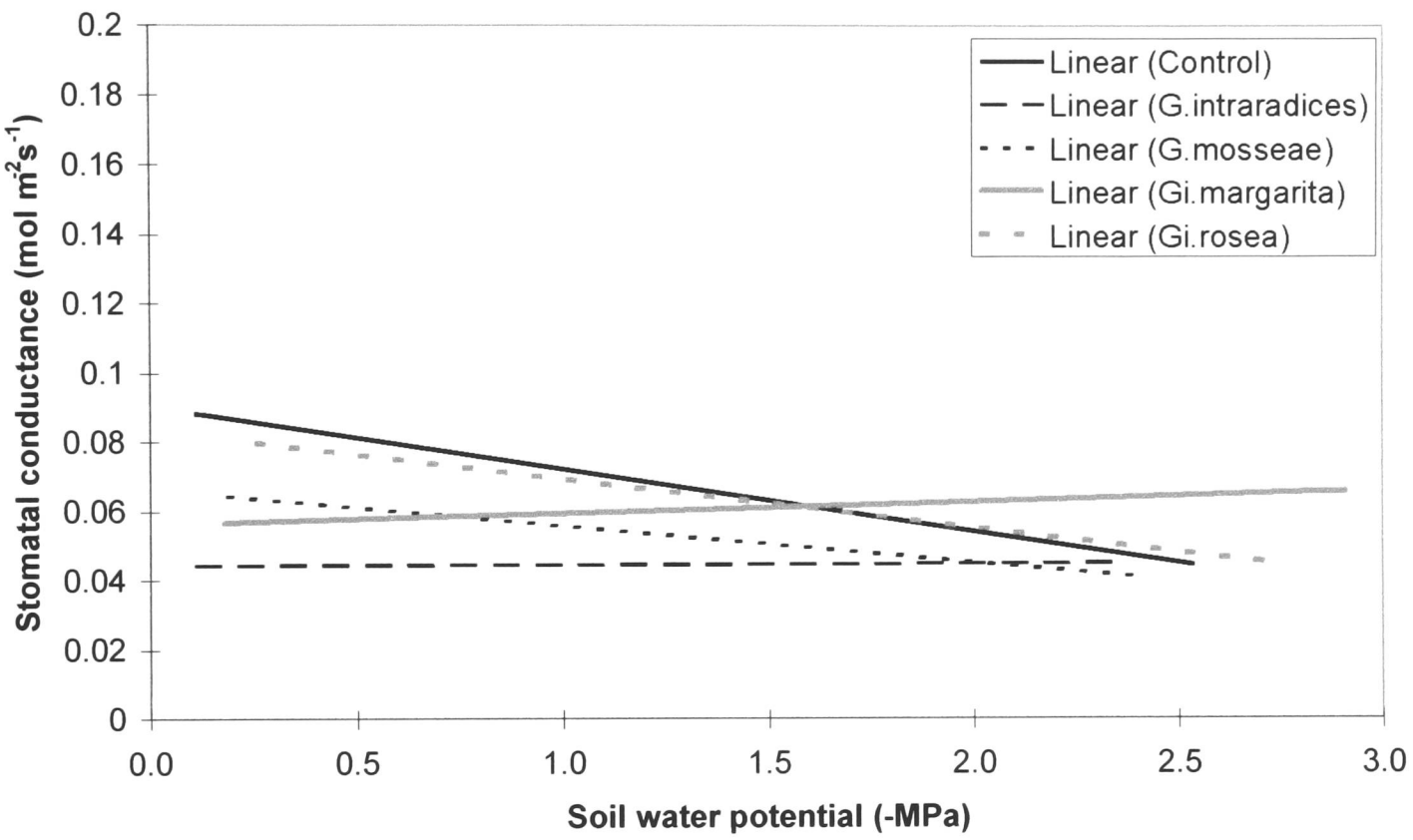
There was greater variation at low water potential but no other pattern to variation. Greater variation was seen in the third period.

The range in soil water potential to which the plants were subjected was similar for three periods. Only during the third period was it four times greater. It is during this third period, and the first period in the first 3 months after inoculation, that a significant relationship was generally seen, with the decrease of water use with decreasing water potential. It was found that this relationship varied during the course of the experiment. There was a greater change in water use with changing water potential in early stages. Later plants showed a decreased responsiveness, with a smaller change in water use with water potential. Water use was also maintained at much lower soil water potentials than previously. Only plants infected with *G.mosseae* and *Gi.margarita* showed a significant change in their response to drying during the four periods. In *G.mosseae* infected plants there was an increasing ability to maintain transpiration at lower water potentials. This was also seen in *Gi.margarita* for periods 1-3. In the fourth period it declined again.

3.3.6 Shoot responses related to soil water potential

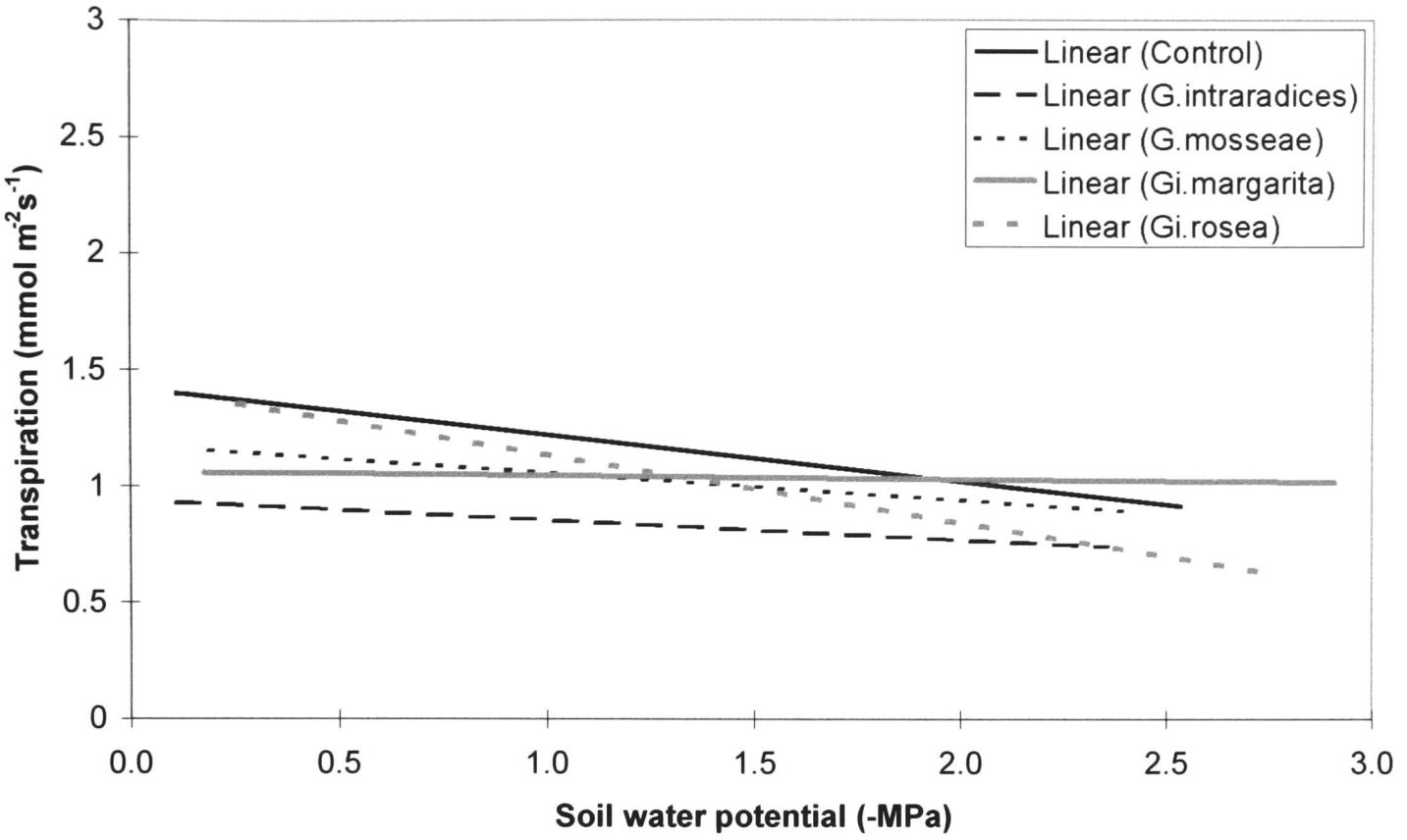
Multiple linear regression was used to analyse the response in terms of stomatal conductance of the different mycorrhiza to changes in soil water potential (Fig.3.14). There was no significant difference found either between mycorrhiza species, or with decreasing water potential.

Fig.3.14 Stomatal conductance compared with soil water potential when inoculated with different mycorrhizal species. Trend lines are fitted by linear regression



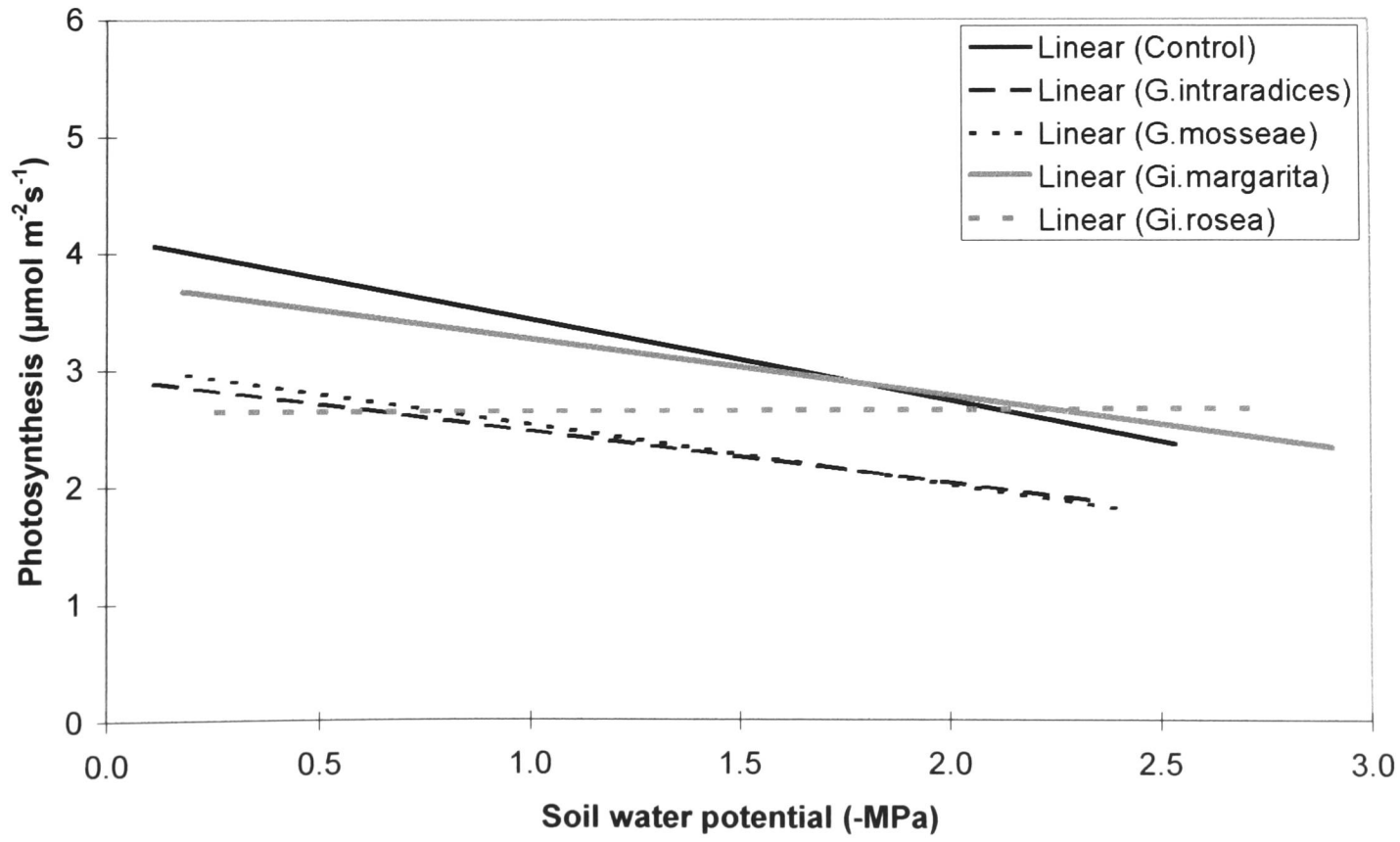
Multiple linear regression was used to analyse the response in terms of transpiration of the different mycorrhiza to changes in soil water potential (Fig.3.15). There was no significant difference found either between mycorrhiza species, or with decreasing water potential.

Fig.3.15 Transpiration rate compared with soil water potential when inoculated with different mycorrhizal species. Trend lines are fitted by linear regression



Multiple linear regression was used to analyse the response in terms of photosynthesis of the different mycorrhiza to changes in soil water potential (Fig.3.16). There was no significant difference found either between mycorrhiza species. However there a strong relationship between photosynthesis and decreasing water potential, significant at <1%.

Fig.3.16 Photosynthesis rate compared with soil water potential when inoculated with different mycorrhizal species. Trend lines are fitted by linear regression



3.4 Discussion

There were indications of some influences of mycorrhizal colonisation on the water use and nutritional status of host plants. The quantity of water transpired, or water use, was broadly related to the leaf area. Leaf area was increased in mycorrhizal plants relative to non-mycorrhizal, although this difference was not significant. The rate of transpiration was generally decreased in mycorrhizal plants. Photosynthetic rate was also decreased in mycorrhizal plants. However the dry matter accumulation varied between mycorrhizal species. Mycorrhizal plants appeared to allocate greater resources to root growth. The response to drying was not shown to be altered by association with different genera and species of mycorrhizal fungi.

3.4.1 Water use

There was no consistent difference in plant water use when colonised with different mycorrhizal species. As the plants aged different species of mycorrhizal fungus had an influence on the response to drought, *Gi.rosea* in the first months, and *G.intraradices* when the plants were older. Mycorrhizal colonisation particularly by *Gi.rosea* increased the root length of the host plants relative to uninoculated plants, possibly due to increased branching in the root system architecture (Hooker *et al.* 1992), although root architecture was not examined.

Plants with large leaf areas usually transpire more than those with smaller areas. However the decrease in transpiration accompanying reduced leaf area may be partially offset by increased exposure of the remaining leaves, resulting in increased transpiration per unit leaf area, even though the total transpiration is reduced (Parker 1949). An increase in leaf area may also be accompanied by a decrease in stomatal conductance, so that transpiration achieves a maximum rather than increasing linearly (Meinzer and Grantz 1991). Small leaves also have greater boundary layer resistance. Water use in this study was generally positively related to leaf area, when transpiration was high. There appeared to be a smaller change in the water use with leaf area in control plants than mycorrhizal plants. Only plants associated with *Gi. rose* did not show this greater increase in water use with leaf area seen in other mycorrhizal plants. This suggested a difference in sensitivity of response to drought

stress between mycorrhizal associations. At the end of a watering cycle when the supply of water was limited, water use was minimal, as would be expected at the end of a drying period, regardless of the leaf area. *G.intraradices* showed a decline in water use with increased leaf area. Plants associated with *G.intraradices* also had relatively lower root mass compared to the other mycorrhizal associations. It may be that plants with larger leaf areas experienced high drought stress and shut stomata sooner. In this case plants with smaller leaf areas would show greater water use.

3.4.2 Gas exchange

Gas exchange was shown to be slightly lower in mycorrhizal than non-mycorrhizal plants. The range in leaf stomatal conductance was more variable in mycorrhizal plants relative to control plants. This is in contrast to the study of Subramanian *et al.* (1995) who also used *G.intraradices* as the mycorrhizal species for drought stress experiments. During drying cycles in maize, they found higher transpiration rates and higher stomatal conductances in mycorrhizal plants. The higher values of stomatal conductance in mycorrhizal plants in their study, indicated that AMF infected plants were able to keep their stomata open for longer than in non-mycorrhizal plants. In studies carried out by Allen (1982) mycorrhizal plants also showed increased stomatal conductance and increased transpiration. Allen and Boosalis (1983) studying wheat colonised by *G.mosseae* and *G.fasciculatum* showed higher stomatal conductances in mycorrhizal plants

There was a noticeable difference in host plant response to soil drying depending on the period of measurement. The periods corresponded to different ages of the plants and to different seasons. A more noticeable dependence of mycorrhizal plant transpiration on soil water availability was seen during the summer when air temperatures were higher and soil water potential was at a minimum. Young plants would be expected to be highly sensitive to drying, particularly in cuttings, where there is a large shoot surface area with a small root system. At this stage mycorrhizal colonisation is low and might therefore not be expected to exert a major influence on the host plants. Alternatively, the small root system might be more highly influenced by even low colonisation. In older plants, the maximum rate of transpiration increased

in mycorrhizal plants. Minimum transpiration also appeared to continue at a much lower water potential. This might be expected from older plants with a larger root system. However a conclusive influence of mycorrhizal infection was not apparent; the largest difference in response, shown by *Gi.rosea* and the control, did not approach statistical significance. Shrestha *et al.* (1995) worked on satsuma trees colonised by *Glomus ambisporum*, *G.fasciculatum*, *G.mosseae*, and *Gigaspora ramisporophora*. In August at high temperatures photosynthesis and transpiration were greater in mycorrhizal trees than non-mycorrhizal, but when air temperature decreased these parameters did not differ significantly. This was explained by larger leaf area, higher shoot P concentration and more vigorous growth seen in mycorrhizal trees. Leaves of mycorrhizal and non-mycorrhizal had similar assimilation rates of CO_2. The difference in amount of photoassimilates was because of the bigger leaves in the mycorrhizal trees. However the current study showed differences in gas exchange, yet few significant differences in nutrient status between mycorrhizal and non-mycorrhizal trees. Similarly little differences in transpiration rates was found by Nelsen and Safir (1982) between mycorrhizal and non-mycorrhizal plants.

In conditions of low radiation and cooler air temperature, leaves transpire more slowly. Drought stress occurs more slowly in the plant, over a longer period of time. Mycorrhizal influence may be more important when the plant is under different degrees of water stress. If water deficit is less severe, differences between mycorrhizal and non-mycorrhizal plants may not be shown. Under these conditions, the gradient in water potential between the soil, root and leaf are less. Consequently the response of the plant may be less.

Any changes in carbon dioxide uptake will ultimately be reflected as a difference in dry matter accumulation. The difference in gaseous exchange between mycorrhizal plants relative to non-mycorrhizal can give rise to higher water use efficiency (g dry matter accumulated kg^{-1} water evapotranspired) (AlKaraki and Clark 1998). This would lead to a higher mycorrhizal plant weight. However increased weight of the root system and shoot was only shown in some mycorrhizal associations, in the present study.

3.4.3 Biomass

The association between host plant and fungus has costs and benefits for the plant. The fungal partner obtains carbohydrate from the plant, and supplies nutrients, particularly phosphate to the plant from the soil. For much of a plant's life phosphorus may not be limiting growth. The mycorrhizal association may only offer benefit at particular times in the life-cycle, such as seedling establishment. Infection might do away with the need to establish an early root system, with the resource costs that entails. Alternatively since soil moisture content directly affects diffusion coefficients in soil, it might be only in dry periods that mycorrhizal transport of phosphate is beneficial. Since hyphae are narrow (less than 5 μm) their cost in carbon terms is no greater then root hairs, and they extend further into the soil. The fungus may consume 5-10% of the total photosynthate (Snellgrove *et al.* 1982, Koch and Johnson 1984).
In this research project, when the biomass of the shoots was compared with the proportion of mycorrhizal colonisation of the roots, a trend was seen where colonisation with *Glomus* species led to increased shoot growth with increasing mycorrhizal colonisation. The reverse trend was seen where colonisation was with *Gigaspora* species. This suggested that *Glomus* had a beneficial effect on the ability of colonised poplar plants to accumulate dry matter under drying conditions, whereas *Gigaspora* appeared to have a negative or parasitic effect. Under conditions of adequate phosphorus nutrition, such as in this study, mycorrhizas have been shown to have negative growth effects in tree crops, because the fungi do not benefit nutrient acquisition and yet continue to draw on the host carbohydrate supply (Graham and Eissenstat 1998). Similar trends were seen in the root biomass, of *G. mosseae* and *Gi. margarita* associations, but not seen with *G. intraradices* and *Gi. rosea*. *Gi. rosea* associations had a greater root length, amongst the lower root weights and the lowest shoot weights than other plants. This mycorrhizal species appeared to be causing the greatest exploration of the soil, and least dry matter accumulation. *G. mosseae* and *Gi.margarita* associations had more moderate biomass accumulations. *G. intraradices* appeared to cause greater allocation of resources to the shoots. These responses confirmed results from other studies. Root and shoot mass have both been shown to be higher in mycorrhizal plants relative to non-mycorrhizal plants (Subramanian *et al.*

1997). Increased biomass in mycorrhizal plants was also found by Ellis *et al.* (1985) using two *Glomus* species in wheat, by Nelsen and Safir (1982). However mycorrhizal plants showed greater dry shoot weights, but no difference in root weights or volumes in other studies which contrasted with the current study (Safir *et al.* 1972, Frey and Ellis 1997). Some mycorrhizal plants have shown no significant difference in leaf areas or root lengths from non-mycorrhizal plants (Allen 1982), and equal dry weights (Allen and Boosalis 1983).

Changes in the allocation of resources to the root system due to mycorrhizal colonisation may allow a host plant to more effectively acquire water during drought stress. In this way the loss in carbon acquisition which occurs during drought can be offset by association with the mycorrhizal fungus. A decrease in shoot and root dry matter and in total root length, due to drought stress conditions was partly overcome by mycorrhizal infection with *G.monosporum* in wheat (AlKaraki and Clark 1998).

The importance of timing in the influence of mycorrhizal fungi on the water relations of plants was emphasised by Lapoint and Molard (1997), who showed that mycorrhizal fungi decreased root growth, and were a greater carbohydrate sink than roots during slow growth of ephemeral plants. However when growth was rapid it was greater in mycorrhizal plants. The mycorrhiza may also have been more important for water rather than nutrient supply where there were short periods of extreme drought stress. This was consistent with the greater differences seen in the response of mycorrhizal species to soil drying, during periods of more rapid water loss in this research. It may have been due to differences in the allocation of resources to the root system.

3.4.4 Nutrition

The nutrient analysis of the plant material was necessary to examine the relationship between drought response of mycorrhizal plants and their altered nutrient status when associated with these fungi. A consistent difference in nutrient status between the mycorrhizal species might have suggested this as a cause of changes in plant response to water deficit stress. However, there was relatively little difference in nutrient status between mycorrhizal and non-mycorrhizal plants, with only potassium

and magnesium showing any significant differences. Few other studies have examined a range of nutrients.

There was no significant difference in phosphorus status between treatments in the current study. This was despite the fact that no additional nutrients were supplied to uninoculated plants, as has been the case in other studies. This result supports the work of Allen and Allen (1986), who found that end-of-season leaf phosphorus concentration did not change with infection. This suggested that phosphorus nutrition was unlikely to be responsible for differences in water relations. AlKaraki *et al.* (1998) also found no difference in phosphorus content of mycorrhizal plants. However it conflicts with other studies in which it was suggested that lowered resistance to water transport in mycorrhizal soybean was due to the improved nutrient status of the plants, relative to non-mycorrhizal plants (Safir *et al.* 1972, Frey and Ellis 1997). In particular the phosphorus concentration was higher in mycorrhizal plants. Nelsen and Safir (1982) found higher tissue phosphorus levels in mycorrhizal plants in an onion and *G.etunicatus* association. It was concluded that phosphorus nutrition was the major cause of differences in drought tolerance, as an indirect effect of nutrient status. Subramanian *et al.* (1997) studying maize with infection by *G.intraradices* also found that mycorrhizal plants had higher phosphorus contents. This helped the mycorrhizal plants to tolerate moderate drought stress and recover to non-stressed leaf water potentials after irrigation.

Mycorrhizal plants have also shown improved nitrogen assimilation (Subramanian and Charest 1998, Azcon and Tobar 1998). They have shown greater uptake of amino acids, serine and aspartic acid, than non-mycorrhizal plants, although the uptake rates were lower than that of nitrate (Cliquet *et al.* 1997). There was greater incorporation of nitrogen into amino acids in the shoots of mycorrhizal plants, in particular glutamine, glutamate, alanine and γ-aminobutyric acid (Faure *et al.* 1998). However increased nitrogen assimilation was not found by Hodge *et al.* (2000), nor any difference in the form in which nitrogen was taken up.

Other nutrient concentrations are generally higher in mycorrhizal plants especially under drought conditions (AlKaraki and Clark 1998). In an association of *G.intraradices* and soybean, colonisation increased shoot and shoot zinc

concentrations (Frey and Ellis 1997, AlKaraki *et al.* 1998). In contrast zinc concentration was reduced in this study.

Similar shoot manganese concentrations have been shown in mycorrhizal and non-mycorrhizal plants (AlKaraki *et al.* 1998). A decrease in manganese particularly in *Glomus* species, in this study, was not found to be significantly different from control plants.

The increase in iron was confirmed by this study, in agreement with AlKaraki *et al.* (1998).

Higher copper concentrations in mycorrhizal plants (AlKaraki *et al.* 1998) were not confirmed in this study.

The uptake of calcium and magnesium in mycorrhizal associations has been examined in ecto-mycorrhiza (Kuhn *et al.* 2000). It was suggested that there was an apoplastic pathway for divalent cations in the root cortex. The endodermis was a significant barrier to the passage of magnesium and calcium ions into the xylem, which was overcome by mycorrhizal colonisation. Given the proliferation of fungal tissue within the roots in AMF associations it is likely that these fungi are also able to alter the pathway of uptake of calcium and magnesium in arbuscular mycorrhiza. This may have given rise to the differences in and magnesium and potassium concentrations in the shoots of mycorrhizal and non-mycorrhizal plants in this study.

3.4.5 AMF species effects

Ruizlozano *et al.* (1995) compared the effect of infection with different *Glomus* species on the drought tolerance of lettuce in a time-course experiment. Photosynthesis, water use efficiency, transpiration and stomatal conductance were assessed. The soil water potentials experienced were higher than those in this study, between -0.06 and -0.17MPa. The responses measured were greatly influenced by choice of *Glomus* species. Leaf area was reduced by drought conditions more in plants infected with *G.occultum* than in those with *G.deserticola*. There was a greater influence of AMF species after drought than before drying. Plants infected with *G.etunicatum*, *G.mosseae* and *G.occultum* had a great sensitivity to moderate stress, whereas those infected with *G.fasciculatum*, and *G.deserticola* only showed

decreases at high water stress. This suggested that different endophyte species have differing abilities to protect the host against drying. The cause of this effect may not be simply ascribed to one mechanism, or to the colonising ability of endophytes. *Glomus* species vary in relation to their effect on the host during drought stress, which was confirmed by the different responses of plants infected with *G.intraradices* and *G.mosseae* in this study, particularly over the course of time. Different responses in hosts infected with *Gigaspora* and *Glomus* species were also shown in their effects on root and shoot carbon allocation and drought response, although they were not significantly different. In the study of AlKaraki *et al.* (1998) wheat inoculated with *G.mosseae* or *G.monosporum* showed differences in shoot and root dry matter increases in the mycorrhizal plants. Plants inoculated with *G.mosseae* showed higher shoot but not root dry matter increases than those inoculated with *G.monosporum*. This difference in carbon allocation response between mycorrhizal associations was similar to the current study.

CHAPTER 4

Control of the water potential of the root-fungal environment

4.1 Introduction

The water potential of a micro-organism cell in the soil is likely, because of its small size, to be in near equilibrium with that of its immediate environment. It is difficult to maintain a constant, uniform water potential in a soil, because of the heterogeneity of its pores. Withholding water as a means of imposing drought can lead to variability in the levels of drought within a population growing in that soil. This means that if studies on the response of fungi to changes in water potential are to be carried out, it may be necessary to alter the soil environment or to use a root medium which is more controllable than soil. It is difficult to control the water potential of soil at a particular level, or to maintain it at that level. For this reason drying cycles are frequently used when studying water deficit stress. A more controlled sustained level of water stress can be achieved by lowering the osmotic component of water potential using chemical osmotica such as polyethylene glycol (PEG). This chapter concerns the response of arbuscular mycorrhizal plants to particular degrees of water deficit stress. This information would be useful in order to assess whether arbuscular mycorrhizae are of particular benefit to the host plant at particular degrees of water deficit stress. For this the control of the rooting environment at specific levels of water potential is required. However if greater control of the environment around the root and hyphae is needed, it may be necessary to make use of a different rooting medium than soil. A more homogeneous medium is desirable.

It was considered that growth of the host plant with its associated fungus would be easily controlled in nutrient solution culture. It was necessary before further work could begin, to test the response of arbuscular mycorrhizae to nutrient solution culture. Arbuscular mycorrhizae are aerobic fungi and require adequate aeration of the rooting environment. Growth in solution should then be possible given adequate aeration. A trial was carried out to test whether the appropriate conditions for the

colonisation and growth of arbuscular mycorrhizae in nutrient solution could be achieved.

This trial was followed by further work to test the possibility of controlling the water potential around the plant-fungus system. This is achieved in solution culture, by controlling the osmotic potential of the solution. Osmotic potential is a component of total water potential in soil. Osmotic potential can be altered using sugar compounds, salts, or polyethylene glycol compounds. Polyethylene glycol compounds have the advantage over the other compounds in that they are not potential natural metabolites of the plant. In addition lower osmotic potentials can be achieved than with sugars and salts. However their disadvantage is that they may have deleterious effect on plants. Some of the smaller PEG compounds may pass into the plant root, and be transported into the transpiration stream, where they may be phytotoxic (Maklon and Weatherley 1965). These difficulties could also be encountered with fungal hyphae.

It was attempted to separate the root-fungal environment from the PEG solutions with semi-permeable membrane. This has been successful achieved in previous studies discussed in section 4.5. If this method could be used successfully in this study, it would be employed in further experimentation with plant-fungal associations, to test their response to particular imposed water potentials.

Experiment (A) Survival and colonisation of arbuscular mycorrhiza with a host plant in solution culture.

4.2 Introduction to solution culture

Nutrient solutions are useful for plant experimental work because the availability of nutrients to the plant can be very precisely controlled. In addition, if the plant can be grown in nutrient solution alone, in a soilless culture, this creates an environment where the whole of the root system has equal access to water and nutrients, without the difficulty of extracting them from soil pores. Because of this more homogeneous environment, much of the experimental work for this chapter involves using soilless cultures. Eight different nutrient solutions were compared by Smith *et al.* (1983) for the growth of maize, ryegrass and clover in silica sand, and showed large differences in their ability to support these species. Long Ashton and Hoagland's no. 2 solution, were both among the most successful solutions for a range of plant species.

It is also useful to grow experimental plants which have been inoculated with arbuscular mycorrhizal fungi in nutrient solutions, for the same reasons as given above. By this method it may be possible to explain whether mycorrhizal fungi can limit drought stress in their host, by greater exploitation of the available moisture in smaller soil pores than the plant roots, or whether the host roots themselves are made more efficient in water uptake. The production of mycorrhizal plants in nutrient solution culture has been studied by Mosse and Thompson (1984), and Thompson (1986). Thompson (1986) grew wheat and maize in sand with varying concentrations of Hewitt's solution and four ratios of NO_3 to NH_4 as a nitrogen source. In general the lowest concentrations of solution gave rise to the most highly colonised plants. Mosse and Thompson (1984) used a system where there was a continuous flow of nutrient solution, based on Hoagland and Arnon (1938), over bean roots in a shallow basin. This allowed adequate aeration of the solution. The pH and nutrient composition was carefully regulated. The quantity of inoculum and the source of phosphorus were varied. There was good infectivity even with small quantities of inoculum. There appeared to be some variation in the infection level depending on the source of

phosphorus, with different levels of solubility. These were monocalcium phosphate, bonemeal, and rock phosphate.

Previous methods in solution culture

Approaches to soilless culture include static hydroponics (Millner and Kitt 1992) flowing hydroponics (Howeler *et al.* 1982, Mosse and Thompson 1984), aeroponics (Hung *et al.* 1991) and *in vitro* with transformed roots (Becard and Fortin 1988). The term hydroponics has been used to described both systems where plants are supported in sand or gravel, with nutrient solution trickled through the medium, and those where plants are supported above a nutrient solution, with their roots bathed in the solution. Hoaglands and Long Ashton solutions appear to be the most frequently used nutrient solutions. Hoaglands solution is suitable for a range of plant species. It lacks ammonium salts which alter pH, and provides micronutrients. Long Ashton solution is frequently used where it is necessary to alter the nitrogen compound and its concentration. A comparison of nutrient solutions for growth of plants in sand culture (Smith *et al.* 1983) showed variations in plant response, particularly iron deficiency. However it showed that plant species requirements are also variable, and solutions may have to be tailored to specific requirements. The use of nutrient solutions for AMF has been studied by a number of authors, described below.

Hydroponics with solid media

Sand supplied with Hoaglands nutrient solution was successfully used by Millner and Kitt (1992) to culture *G.etunicatum*, *G.mosseae* and *Gi.margarita* in corn. Full and half strength nutrient solution were supplied using an intermittent drip system. The availability of nitrogen in a sand medium has also been successfully modified when supplied with nutrient solutions of varying N concentration. The source of nitrogen was also altered as a nitrate or ammonium compound (Thompson 1986).

Hydroponics without solid media

Mosse and Thompson (1984) grew beans with mycorrhizal colonisation in trays in which roots were in recirculating nutrient solution (Nutrient film technique). Inoculum of 0.05g per plant gave 5-10% colonisation after 6 weeks. Nutrient levels in excess of requirement were circulated in a large volume of water to avoid the need for monitoring in commercial culture. The flow rate was 1 l min^{-1} in a 1 mm deep film of solution. The shallow film of solution ensured adequate aeration. The host plants were inoculated before putting into solution with *G.mosseae* and *G.fasciculatum*. Hoaglands solution was adjusted to pH7 for *G.mosseae* and pH6.3 for *G.fasciculatum*. Full, ¼, 1/10, and 1/20 strength solution were compared in their effects on AMF growth and plant health. At full strength manganese toxicity in the plants was apparent. The percentage infection of AMF was generally highest in 1/10 strength solution. Plants were chlorotic in 1/20 strength solution. AMF infection levels in root samples were variable particularly with root age. Infection was greater in older brown roots (30-60%), although some were already infected before. In the younger roots the spread of infection was seen from 0-10%. AMF infection appeared to be anatomically normal. More internal mycorrhizal structures in roots were seen at low solution concentrations, whereas external fungal structures were more evident at high solution concentrations. It was suggested that this effect could have been due to root death caused by nutrient toxicity. The proportion of internal and external structures was balanced at mid-range concentrations.

Howeler *et al.* (1982) increased the rate of flow of the solution to 1.6 l min^{-1}. In slow-moving solution the P concentration will change around the roots. In fast-flowing solution, the extent of depletion at root surfaces would be reduced in comparison with soil systems. The solution was also replaced every 7 to 10 days. A substantial positive effect of mycorrhizas on P uptake and growth persisted under conditions of moderately severe P deficiency. This suggested that the beneficial effects of mycorrhizal colonisation on plant P nutrition may not be solely due to overcoming the limitation on P uptake imposed by slow diffusion of phosphate through soil. Fungal hyphae may have a greater ability to absorb P at very low external concentrations than roots. Alternatively it may be the greater absorptive surface

provided by hyphae. The levels of root infection were lower than typically found in soil. This may have been due to removal of root exudates from the root surface by the flowing solution.

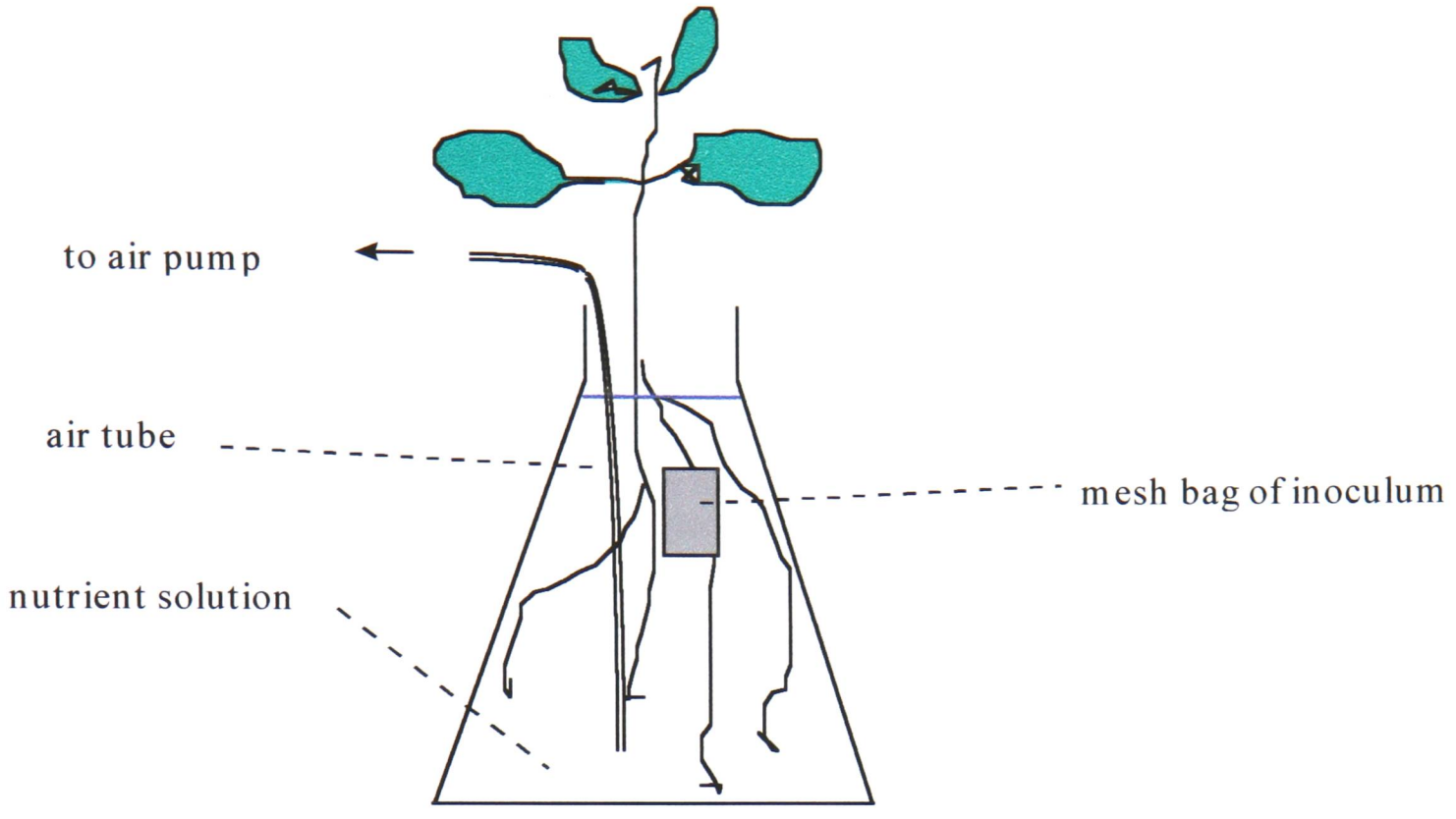
The aim of this experiment was to determine whether AMF could survive in solution culture. This experiment was carried out in order to test the capacity of arbuscular mycorrhizal fungi to survive and grow in aqueous culture. This was necessary because further experiments would involve the use of the osmoticum polyethylene glycol (PEG) in solution to examine water uptake by plants colonised by arbuscular mycorrhiza.

4.3 Materials and Methods

Wild Cherry (*Prunus avium*) plants, grown from micropropagated plantlets (HRI, Efford), were set up in 280ml of 1/4 strength Hoaglands nutrient solution in 500 ml conical flasks (Sigma) (Fig.4.1). There were six replicates. The plants were supported in the neck of the flask by wrapping in cotton wool. The flasks were covered in aluminium foil to exclude light from the nutrient solution. The solution was aerated by passing 0.5 mm diameter plastic tubing (Aquatec) into the bottom of each flask. They were each connected via valves to an air pump (Rena). The valves were adjusted so that each one supplied air at the rate of 2 bubbles per second. This was sufficient to maintain movement of the solution without disturbance of the plant root system. Bags with dimensions of 6x4 cm were made from 0.5 mm weave nylon mesh (Plastok Associates). They were filled with chopped roots of cucumber cv. Bush Champion colonised with *Glomus intraradices* FL-208-4. The mesh bags were each threaded onto a wire and attached around the stem of the plant so that they hung next to the root system.

The nutrient solution was replaced completely each week to prevent the accumulation of algae and to supply the same concentration of nutrients. On a daily basis the flasks were refilled to 280 ml with deionised water. The plants were kept in a glasshouse with a temperature range of minimum $10^{O}C$ to maximum $28^{O}C$, and photosynthetically active radiation of 100 to 450 $\mu mol\ m^{-2}s^{-1}$.

Fig.4.1 Construction of solution culture equipment



Roots were sampled after 8 days, 4 and 8 weeks. They were collected from midway down the root system, near the inoculum bag. They were cleared and stained, and then examined under a dissecting microscope (Wild M10, Leica) for mycorrhizal colonisation. The methodology for this is found in Chapter 2. Results are presented in Table 4.1. After 11 weeks the plants were harvested completely. Structures were identified in the root samples which included external hyphae of mycorrhiza, internal hyphae and other internal structures such as arbuscles and vesicles. These results are also presented in Table 4.1.

4.4 Results and Discussion

After 8 days it was observed that there was active plant root growth. New white root tips were visible. At this time no hyphal growth could be observed. It was not until plants had been cultured in solution for 4 weeks, that new fungal hyphae could be seen inside the roots. Fungal growth was highly variable between plants. In the subsequent samples, hyphae were seen to extend and grow out into the nutrient solution. The change in root colonisation at each sampling time suggested that there was an acclimation period when the fungus was transferred from a soil to a liquid environment, seen as a drop in colonisation at the 8 week sampling point (Table 4.1).

Table 4.1 Survival of *Glomus intraradices* in aerated Hoaglands nutrient solution.

Time in solution (weeks)	4	8	11
% infection	47.8	9.4	37.0
S.E.	15.2	5.4	7.1
	a	a,b	b

where a =internal hyphae
b =external hyphae

Hyphae could be seen proliferating in the nutrient solution indicating that the production of external mycelium was not inhibited by the solution culture. Colonisation was also comparable with AMF colonisation in soil (see Chapter 3).

Growth in solution culture was considered successful, and could be used in further experimentation.

Experiment (B) Use of Polyethylene Glycol to alter the water potential surrounding plant roots.

4.5 Introduction to osmotica

In nutrient solution cultures where there are no soil pores, or in silica sand where they are of a limited range in diameter, an alternative method is required to control and vary the matric potential of the substrate. Polyethylene glycol has been used for many years to fulfil this role. Although this method is far removed from the natural system of wetting and drying in soils, it does have the advantage of precise, repeatable control of the potential of the substrate. Polyethylene glycols (PEG) of various molecular weights have been used, to examine a variety of processes including, plant growth (Larson and Palashev 1973), turgor pressure and cell expansion (Blake *et al.* 1991, Hohl and Schopfer 1992), xylem cell wall metabolism (Whitmore and Zahner 1967), abscisic acid accumulation and electrolyte leakage during drought stress (Tan and Blake 1993, Reid 1974). The variation in drought tolerance between varieties or species has been tested (Nguyen and Lamant 1989, Perry *et al.* 1978, Leustek and Kirby 1990), and seed germination relative to soil water potential (Kaufmann and Ross 1970).

Water deficit is applied to roots by lowering osmotic potential of nutrient solution. In this way the heterogeneity in water potential that characterizes a solid substrate and the water potential gradient between roots and environment that exist when plant is raised in soil, should be avoided (Fiscus 1972). The effect of PEG on plant water relations as compared with plants growing in soil has been examined in a number of studies. PEG 6000 appears to give rise to similar water relations as those expected in soil at the same water potentials (Kaufmann and Eckard 1971, Nguyen and Lamant 1989). The pressure potential in the root and leaf decreases in PEG 6000 solution, correlated with the osmotic potential of the solution, while the osmotic potential of the root xylem sap remains constant. In a PEG 400 solution, however, there is a decrease in the osmotic potential of the root xylem sap, due to the increased accumulation of K^+, Na^+, Ca^{2+}, and Mg^{2+} ions and possibly other compounds in the xylem. This decrease in osmotic potential allows the entry of water and causes

guttation. The importance of positive ions and sugars in influencing the osmotic potential was also found by Premachandra *et al.* (1989). This suggests that PEG of low molecular weight is less suitable for water relations studies than higher molecular weights, such as PEG 6000. Lawlor (1970) examined the absorption of PEG by plants, and found toxic effects on plant growth using both low and molecular weight PEG solutions. These were not due to impurities in the PEG solutions, or to the detergent properties of PEG (Whitmore and Zahner 1967), lowering the surface tension within the leaf, which affects cell permeability. PEG 200 and mannitol were able to enter plant roots, which altered the osmotic potential of the xylem sap or in the leaf. PEG of 1000 molecular weight or above entered roots more slowly, unless the root were damaged, when entry was increased. It was suggested that high molecular weight PEG causes blockage of the transpiration pathway, reducing water absorption and causing desiccation of the plant. Two high molecular weight PEG solutions, 6000 and 20000, were compared by Williams and Shaykewich (1969), and compared to the pressure membrane technique of measuring soil water potential. No significant difference was found between these methods. This suggests that any high molecular weight PEG solution could be used to control the potential of a solution, provided that obstruction of the transpiration pathway is prevented.

Previous methodologies

PEG solutions have been used directly on plant tissues such as cell cultures (Leustek and Kirby 1990), tissue pieces (Whitmore and Zahner 1967) and coleoptile pieces (Hohl and Schopfer 1992).

The PEG solution has also been applied to a solid medium such as vermiculite (Perry *et al.* 1978, Larson and Palashev 1973) or rockwool (Urban *et al.* 1994). Roses were grown in rockwool in a phytotron. Water uptake was estimated from the difference between rate of supply of nutrient solution and rate of leaching, using a flowmeter. Transpiration was measured by recording loss in weight of the whole system.

In order to avoid the toxic effects of PEG, methods have also been devised where the solid medium is separated by semi-permeable membrane. The osmotic

potential of the solution on one side of the membrane is used to control the matric potential of the medium on the other side. This method was used successfully by Williams and Shaykewich (1969). Double thickness visking dialysis tubing separated soil samples from PEG solution. After 6 days equilibrium was established. "Boats" of cellulose acetate membrane filled with soil have also been floated on trays of PEG solution (Kaufmann and Ross 1970). The use of semi-permeable membrane was chosen in this trial to ensure that no polyethylene glycol came into contact with roots or fungal hyphae. Some form of homogenous medium was also required to support the plant.

However the presence of the dialysis membrane may itself influence the achieved potential in the solid medium (Bernier 1995). The matric potential of peat was adjusted in that study with PEG solutions with and without semi-permeable membrane.

Other work with mycorrhizae

Polyethylene glycol has been used for mycorrhizal studies of drought stress but they concerned ectomycorrhizae (Mexal and Reid 1973). These were ectomycorrhiza grown in artificial nutrient media. The PEG was neither metabolised nor absorbed as inorganic salts or sugars can be, suggesting that the method could be adapted for use with arbuscular mycorrhiza, growing in association with host plants.

The aim of this experiment was to determine whether polyethylene glycol could be used to control water potential of the rooting environment.

4.6 Materials and Methods

This experiment was carried out to test the possibility of using the osmoticum polyethylene glycol (PEG) to alter the water potential of a medium. PEG 6000, with a mean molecular weight of 6000, and PEG 20000 with a mean molecular weight of 20000 were tested. Silica sand of 1mm and 2mm particle diameters were used.

Solutions of PEG 6000, a commonly used osmoticum, and PEG 20000 (BDH Merck Ltd.) were made up in deionised water to calculated osmotic potentials (Williams and Shaykewich, 1969). For these potentials the following dry weight of PEG was added to deionised water and stirred vigorously on an automatic stirrer. Gentle heating was required at higher concentrations.

Table 4.2 Osmotic potential obtained solutions of PEG 6000 and PEG 20000, from Williams and Shaykewich 1969

Potential (MPa)	Weight PEG 6000 (g/l)	Weight PEG 20000 (g/l)
-0.1	75	75
-0.3	140	140
-0.5	175	175
-1.0	225	230
-1.5	265	270

The water potential of these solutions was tested with a thermocouple psychrometer. Circles of filter paper (Whatman) were cut out with a hole-punch. One was dipped in a solution with forceps. A thermocouple psychrometer (Wescor, Inc. model HR-33) was used, being read in the dew point mode, to determine water potentials of the solutions. The filter paper soaked in sample solution was placed in the chamber of the microvoltmeter. The system was allowed to come to equilibrium for 15 minutes. A reading was then taken (μV). This was carried out at 16^{O}C in an incubator. Readings were taken every 15 minutes until successive readings were the same. At this point the sample had come into thermal and vapour pressure equilibrium with the chamber. The readings were compared with standard solutions of NaCl for calibration, at concentrations of 0.1 to 0.5 molal. The relationship between the expected and achieved osmotic potential was plotted and is presented in Figure 4.2.

Bags of 1 mm sand and 2 mm gravel were made up with semi-permeable membrane. These were used to represent the rooting medium of future experimental plants. A trial was carried out to determine whether the water potential of the media in the bags could be controlled by placing them in PEG solutions of known osmotic potential. In addition, the potential of the media would be determined by weighing the bags, giving the mass of water within the media. This experiment was carried out twice. The first results are given in Table 4.3.

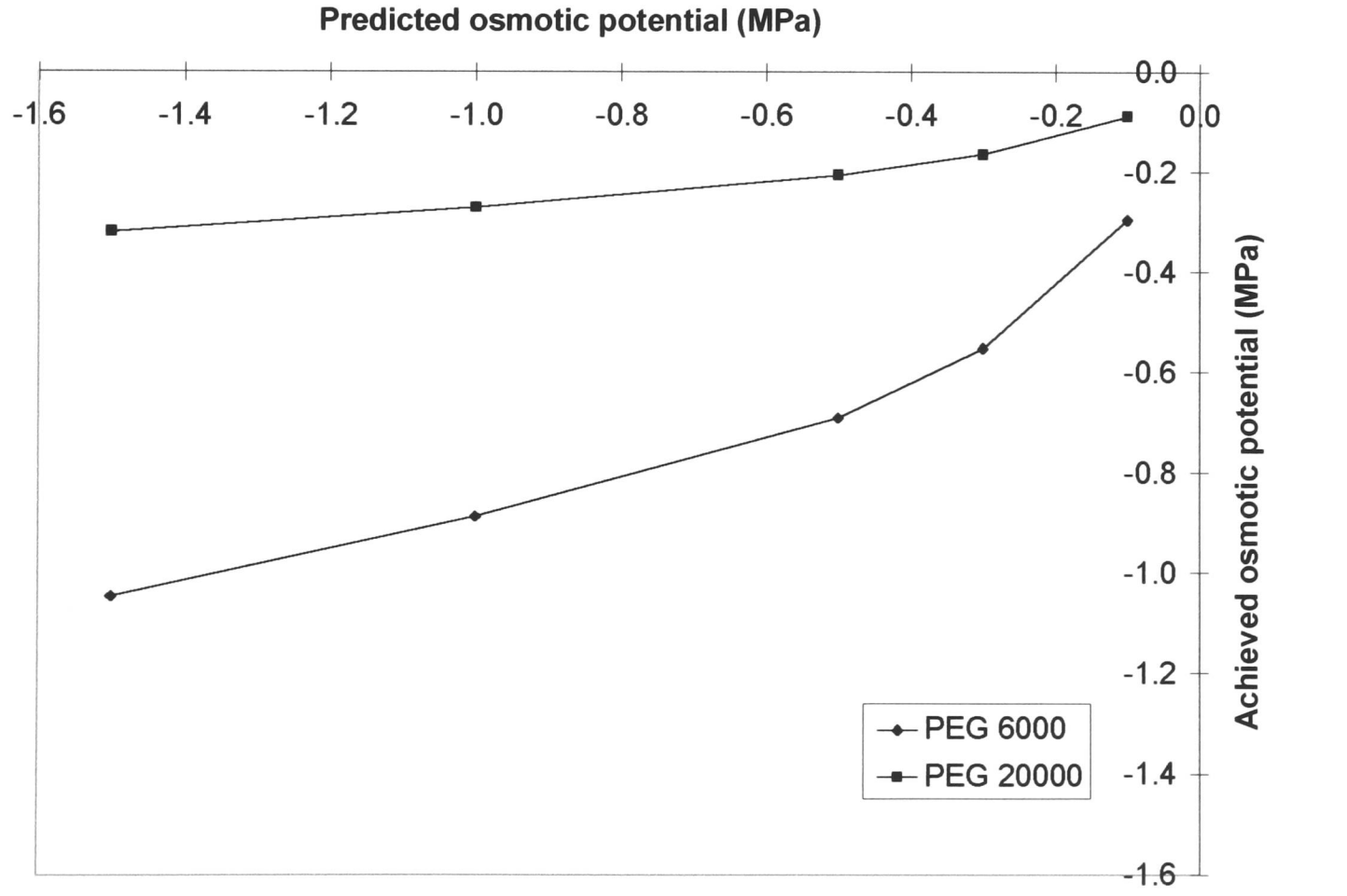
Bags of dialysis membrane (Medicell Ltd.) supplied as tubes, were made by knotting at one end. They were 15 cm in length. The weight of each membrane was found to 0.01 g. The membrane was permeable to substances of molecular weight 12000 or less. It was for this reason that PEG 20000 was added to the treatments, as it would be unable to pass through the membrane. 1 mm particle diameter silica sand was saturated with deionised water and put into the bags. The weight of completely dry sand was 50 g. The bags were sealed at the top with a clip (BDH Merck Ltd.), and weighed again. One each of these filled bags was put into a Magenta pot and covered with a solution of PEG 6000 or PEG 20000 at -0.1, -0.3, -0.5, -1.0, -1.5 MPa. Another set of bags was made up using 50 g dry weight of 2 mm gravel, and also placed in solutions of the same range of water potentials. The treatments were thus two types of medium, sand or gravel, and two molecular weights of PEG, 6000 and 20000, at a range of concentrations.

The weight of the bag was measured after 24 hours. The outside of the bag was gently rinsed using a wash bottle and blotted with tissue paper before weighing. It was replaced in the solution, and then weighed again after 5 days and 6 days. The bags were then all put into deionised water and weighed again after 5 days. This cycle of placing in PEG solution and deionised water was repeated three times.

4.7 Results and Discussion

Polyethylene glycol solutions were made up using PEG-6000 and PEG-20000. The concentrations were calculated to achieve osmotic potentials of -0.1, -0.3, -0.5, -1.0, -1.5 MPa. These concentrations were tested with a dew point microvoltmeter. It was found that these increasing concentrations of PEG were achieving a steady change in osmotic potential with increasing concentration of PEG. However the achieved osmotic potential was less than that expected from previous studies (Fig.4.2). The steady change in osmotic potential which was controllable by changing the PEG concentration, was required to use this osmoticum in further experimentation. Therefore these solutions could be used to control the osmotic potential of the nutrient solution culture.

Fig.4.2 Relationship between measured osmotic potential using dewpoint microvoltmeter, and expected osmotic potential in PEG solutions of varying concentrations.



The change in weight of saturated media was measured after 6 days in PEG solutions of varying concentrations. It would be expected that the media would lose water into the solution outside the bag. They would also be expected to lose more water in solutions of higher PEG concentration. However this pattern cannot be seen.

Some media, particularly in PEG-6000 solution, were even seen to have absorbed water (Table 4.3).

Table 4.3 Weight of water (g) in sand when in PEG solutions

PEG 6000	Osmotic potential	Day0	Day1	Day5	Day6	Total change
	-0.1MPa	11.67	11.89	12.53	12.57	2.45
	-0.3MPa	11.32	14.16	13.78	13.75	3.82
	-0.5MPa	10.18	13.36	13.05	13.00	3.97
	-1.0MPa	9.93	4.16	5.62	5.56	-3.18
	-1.5MPa	11.41	12.09	11.70	11.72	1.51
PEG 20000	Osmotic potential	Day0	Day1	Day5	Day6	Total change
	-0.1MPa	11.96	0.81	1.34	1.17	-8.85
	-0.3MPa	11.16	11.00	10.11	10.36	0.65
	-0.5MPa	11.09	9.00	8.85	9.20	0.03
	-1.0MPa	11.44	1.38	0.55	0.62	-8.94
	-1.5MPa	10.66	1.47	0.59	0.76	-8.09

The experiment was repeated over a longer period. All the bags were placed in deionised water. Over the course of 5 days, it could be seen that the media came to a relatively consistent weight, except for two bags. The bags were transferred to the PEG solutions described above, to test again whether they would lose water in the expected pattern. Again this was not seen. In contrast, the bags were again found to have gained weight, indicating that they had gained water (Table 4.4).

Table 4.4 Weight (g) of sand and gravel when alternated between water and PEG solutions

	Day0	Day5	Day6	Day10	Day11	Day15
Sand Treatment	Water	Water	PEG 6000	PEG 6000	Water	Water
-0.1MPa	4.66	12.34	10.41	15.81	15.95	14.55
-0.3MPa	5.50	11.94	9.87	14.05	14.01	13.90
-0.5MPa	10.96	11.81	9.46	13.33	13.47	13.69
-1.0MPa	13.16	13.26	9.83	13.39	12.16	12.35
-1.5MPa	5.59	10.71	8.25	13.23	12.80	13.05
Sand Treatment	Water	Water	PEG 20000	PEG 20000	Water	Water
-0.1MPa	2.51	6.22	4.60	12.84	13.10	13.18
-0.3MPa	10.46	11.91	10.23	14.17	15.52	15.23
-0.5MPa	4.13	10.98	7.71	15.02	16.06	16.06
-1.0MPa	14.36	13.98	11.87	13.57	13.30	13.35
-1.5MPa	3.61	12.80	10.70	14.21	13.85	13.30
Gravel Treatment	Water	PEG 6000	Water	Water	PEG 6000	PEG 6000
-0.1MPa	1.69	2.83	12.02	14.15	13.71	15.37
-0.3MPa	1.68	2.93	2.58	8.73	11.07	12.36
-0.5MPa	2.87	6.42	8.71	12.56	13.18	14.15
-1.0MPa	1.04	4.68	12.37	14.46	12.41	14.05
-1.5MPa	1.86	3.78	5.20	11.66	11.28	12.29
Gravel Treatment	Water	PEG 20000	Water	Water	PEG 20000	PEG 20000
-0.1MPa	1.40	2.91	1.42	6.51	7.81	9.58
-0.3MPa	2.12	2.64	3.48	9.58	6.73	8.24
-0.5MPa	1.05	2.24	0.77	6.65	6.90	8.67
-1.0MPa	0.84	1.95	0.02	5.50	3.26	6.08
-1.5MPa	1.89	2.84	0.86	5.64	6.63	8.74

Table 4.5 Significant difference between substrate and PEG solution treatments.

Treatment	Osmotic potential	Time
sand / PEG 6000	NS	<1%
sand / PEG 20000	NS	5%
gravel / PEG 60000	NS	<1%
gravel / PEG 20000	1%	<1%

Two-factor analysis of variance was carried out on these changes in weight in PEG solutions, for each combination of sand or gravel, and PEG-6000, or PEG-

20000. It was found that the weight changes with time were generally significant at less that 1%. This indicated water movement between the medium and surrounding solution. However the variation between concentrations of PEG were generally not significant. The percentage significant difference for each treatment combination is given in Table 4.5.

It was expected to control the water potential of the medium in the bag, and to relate this to the weight of the bag, as its water content changed. This could be used as a quick, direct method of altering the root environment of a plant. A suitable substrate / solution combination might have been gravel suspended in PEG 20000. There were significant differences in weight of water held in the bags between different osmotic potentials. There were also significant changes when the bags were transferred between water or PEG solution. However there was no regular pattern to these changes, through the range of PEG solutions.

The polyethylene glycol was used to lower the osmotic potential of the solution outside the semi-permeable membrane. Water flows from a high water potential to a lower one. As a component of total water potential, a lower osmotic potential causes a lower total water potential. In this case then, water flows from the inside of the membrane to the solution outside. The additional water dilutes the polyethylene glycol solution causing an increase in its osmotic potential. When its total water potential is equal to that inside the membrane, outwards water flows stops.

There is no solute in the water inside the membrane. The water potential inside the semi-permeable membrane is largely determined by the matric potential component, because of the presence of capillary pores between the particles of sand or gravel. The smaller diameter pore spaces between sand particles give rise to lower matric potential and hence a lower total water potential. The sand bags should then retain water more strongly against the outflow of water, when the bags are suspended in the osmoticum. When the bags are suspended in the osmoticum water flows out until the matric potential inside the membrane is equal in magnitude to the osmotic potential outside. When the bags are suspended in deionised water only, the lower

matric potential inside the membrane causes water to flow into the bags. This continues until the pressure of water inside the bags gives rise to a pressure acting inwards due to the membrane, and tending to prevent the inflow of any further water.

The flow of water between the inside and outside of the membrane is dependent on the gradient in water potential on either side of the membrane. This causes a gradient of water potential between the centre of the substrate within the bag and the edge. The volume of the solid medium was similar to that used by Williams and Shaykewich (1969). Equilibration times were similar in this study and theirs. However constant values for water content in the medium were not found in that time in this study. This indicated that equilibrium between the solution and all of the substrate throughout the diameter of the bag was not being reached.

Movement of PEG 6000 through the dialysis membrane could have caused differences in water movement between PEG 6000 and PEG 20000 solutions. However there was no significant difference between the use of PEG 6000 and PEG 20000 to control osmotic potential. This is similar to the results of Williams and Shaykewich (1969), who compared the use of both PEG 6000 and PEG 20000 and the pressure membrane method.

The use of polyethylene glycol as an osmoticum to control the water potential surrounding fungal hyphae was to be the next stage in this experimental work. However because no pattern could be found in the gain and loss of water in the experimental system, it could not be used further. It was concluded that control of the water potential of the rooting media with PEG could be not achieved, and this method was not suitable for further experimentation.

CHAPTER 5

Use of a two-section rhizobox to examine root/mycorrhizal hyphae interactions in water uptake.

5.1 Introduction

The response of plants to drought stress is often different in mycorrhizal and non-mycorrhizal plants. A number of suggested mechanisms have been put forward to explain this phenomenon. They include altered root system architecture such as greater root branching and hence increased surface area (Berta *et al.* 1990, 1993, Hooker *et al.* 1992). Direct water transport (Faber *et al.* 1991), and increased root water conductance (Safir *et al.* 1972) have been suggested. The plant metabolism has also been studied, including altered carbon allocation (Davies *et al.* 1996), modified root-to-shoot signalling (Auge and Duan 1991), or the indirect benefits of improved nutritional status (Fitter 1988). These are reviewed in Chapter 1. Previously in Chapter 3 the water requirement of plants was examined in response to changes in soil water potential with time. Conventional pot experiments do not differentiate the importance of the internal and external structures of mycorrhizal roots. The external hyphae of AMF can extend into volumes of soil not accessible to the host roots. They are then experiencing different conditions in water availability than the host roots. This chapter examines the effect of spatial variation in soil water availability, experienced by different portions of a mycorrhizal root system.

5.1.1 Experimentation with obligate symbionts

The major difficulty encountered in studies on arbuscular mycorrhizal fungi is the fact that they are obligate symbionts; that is they cannot be cultured in the laboratory independently of the host plant. Experimental studies carried out on mycorrhizae involve both the plant and the fungal partner. The response of the fungus alone to an imposed treatment cannot be tested independently of the plant. A mycorrhizal plant response also cannot be explained as directly due to the treatment, or to an indirect alteration in the plant's physiology as a result of the fungal colonisation. If mycorrhizal plants are compared with non-mycorrhizal plants, as has

been the case in many studies on mycorrhizal colonisation, there are differences in dry weight, root:shoot ratio and plant nutrient status. If the role of mycorrhiza in plant water relations is being studied it is helpful to compare plants of the same size and nutritional status. If mycorrhiza are typical in natural ecosystems, it follows that a plant without associated mycorrhizal fungi will function in a sub-optimal manner. This will be demonstrated most when the system is under stress. In this case it may be useful to consider the effect on the host plant of removal of the external fungal hyphae. This chapter records experiments which remove the external mycelium and monitor changes in the host plant response in this sub-optimal condition. A method for separating the influence of the plant and the fungal partner in experimentation is to make use of specialised plant growth chambers.

5.1.2 Plant growth chambers

A number of different methods have been used in an attempt to separate the effect of mycorrhizal hyphae themselves, and roots with altered physiological responses due to the presence of the hyphae. A range of microcosms have been used, particularly for nutrient studies involving mycorrhizae. Phosphorus nutrition has been studied (Ravnskov and Jacobsen 1995, Amijee *et al.* 1993, Pearson and Jakobsen 1993) and nitrate acquisition examined (Frey and Schuepp 1993, Tobar *et al.* 1994). These microcosms allow mycorrhizal hyphae to grow into a section from which roots are excluded, most commonly using mesh of 20-60 μm diameter pores. The two sections of the microcosm with roots and associated hyphae, and hyphae only, can be treated independently. Microcosms have been adapted for studies on plant-fungal water relations. Work carried out by Hardie (1985) involved removing a significant portion of the external hyphae of mycorrhizal plants and using these as control plants to compare with others with intact mycorrhizal mycelia. In this way all plants were identical except for access to an external mycelium. Transpiration flux was obtained by weighing the plants and sand in Petri dishes and relating it to the leaf area to give a measure of the quantity of water transpired for a given leaf area. Mycorrhizal plants with hyphae removed showed an inability to maintain their transpiration flux. Due to the fact that there were no differences in stomatal resistance at any stage during the

experiment, it was suggested that there was no whole plant response to the imposed treatments such as a change in water potential of the plant tissue. It was suggested that this meant that the drop in transpiration was due to the direct effect of hyphal removal on the resistance of the root. Removing the hyphae increased the root resistance because of the reduced surface area for water absorption (see Chapter 1). There is some difficulty in drawing this conclusion, because there is no comparison with plants which have not undergone a period of drying during transplantation. In addition it is doubtful whether damage to the root system could be avoided, both during removal from the sand and when hyphae were cut from the root.

5.1.3 Plant growth chambers used to control water availability to mycorrhiza

Two studies (Faber *et al.* 1991, George *et al.* 1992) built on the idea of removing the effect of extraradical hyphae by separating the volumes of soil in which the hyphae and roots grew. Boxes were constructed so as to contain two soil compartments separated by stainless steel screens and an air gap. By allowing hyphae to cross a screen or barrier through which roots cannot pass, they can be watered differentially from the roots. The air gap, which need be only 1-3 mm across prevents mass flow or diffusion of nutrients and water through the soil. In this respect the box is similar to the split-root system frequently used in physiological studies to determine the effect of imposing different environmental conditions on sections of a plant root system.

This type of growth container or "rhizobox" has been used to remove the extraradical hyphae of *Glomus clairodeum,* without damaging the host plant, cowpea (*Vigna unguiculata*) roots (Faber *et al.* 1991). Initially, there were rhizoboxes with non-mycorrhizal, and some with mycorrhizal plants. With the hyphae in place in their soil section, the plants were subjected to a drying cycle. The total water transpired from both sets of boxes was measured gravimetrically. It was found to be greater by the mycorrhizal plants. Then during a second drying cycle, the connection between the plant and the extraradical hyphae in some of the boxes, was severed by cutting between the two sections of the box. In this experiment the total water loss of the plants which had had their hyphae severed was intermediate between the loss by intact

mycorrhizal and by non-mycorrhizal plants. Treatments were not significantly different from each other. There was however a significant difference between the mycorrhizal and non-mycorrhizal treatments. The experiment was repeated using an alternative host plant, sunflower (*Helianthus annuus*) and omitting the non-mycorrhizal treatment. In this case there was a significant difference in water loss between those plants where hyphae were severed, and those were it was not. It was suggested that in this experiment that greater numbers of hyphal crossings into the hyphal section could be the cause of the difference. Similar rhizoboxes were used to study the effect of *Glomus mosseae* on the grass *Agropyron repens* and white clover (*Trifolium repens*) (George *et al.* 1992). In this system there was no evidence of direct transport of water by hyphae. Rates of water loss from the hyphal sections were measured using microtensiometers. These showed very low rates of water loss, even during the period of imposed drought. The hyphae were severed from the roots as in the study above, but no change in the rate of water loss was observed from the hyphal compartment. This suggests that evaporation rather than absorption by the hyphae was the cause of the decrease of water loss. The two systems differed in mycorrhizal species, host plants and the dimensions of the boxes, all of which may be the reason for the contradictory results. However these studies suggested the use of rhizoboxes for the current study using poplar cv. Robusta and *Glomus intraradices.*

5.1.4 Adapted use of plant growth chambers

The contradictory results of earlier studies suggested that an alternative approach which could make use of the rhizoboxes was needed. What if the mechanism of AMF effect on water supply was more subtle than this, involving signalling, or transport of some substance within hyphae to plant, which provoked a stress tolerant response. Given the thinness of the hyphae, an average of 5 μm (Abbott and Robson 1985) they would have the potential to act as mini-tensiometers providing information, rather than tiny roots which simply transport water. Are AMF acting as a sensor for the plant, responsive at a particular soil water potential?

Could the rhizobox system be used in the split-pot manner, to test whether plants were able to make use of the hyphae to signal water availability? The situation

of having the mycorrhizal hyphae in a separate section allows rhizoboxes to be used in the same manner as split-root pots. Earlier studies give some indication that mycorrhiza have a greater influence ameliorating plant response to drought stress, rather than improving growth under conditions of adequate soil water. This could suggest an altered signalling response to drought conditions.

5.1.5 Split-root experiments and heterogeneity of the root environment

Split pot experiments examine whole plant response to heterogeneity in the soil environment. This has also been achieved in the field (Green and Clothier 1995). Before preferential irrigation there was a correspondence between root distribution and pattern of water uptake. Partial wetting of the soil on one side of kiwi-fruit led to preferential use of roots in the wet zone, and reduced activity in the dry zone. The sap flow in vines which were wholly or partially watered was comparable, indicating that the partially watered one was able to maintain its transpiration rate even if only a portion of the root system had access to water. When the dry zone was rewatered, previously inactive roots were able to quickly recover their activity. The activity of roots in terms of transport of water can be modified. They are not continually operating at peak capacity. Split pot experiments have been used to examine whole plant responses to differences in the soil environment (Croker 1998). Split-root studies have been carried out previously to examine the amelioration in response to drought stress of mycorrhizal roots (Auge *et al.* 1994). However these studies have compared mycorrhizal roots with non-mycorrhizal roots. It is possible that the hyphae themselves could be responsible for the altered response to drought of mycorrhizal plants. A rhizobox system could be used to test this, by altering the water availability in the hyphal section compared to the root+hyphal section, in the same manner as traditional split-root experiments. The response of the host plant in terms of gas exchange and dry matter accumulation could be examined as a function of the hyphae themselves, rather than mycorrhizal roots. This emphasis on the extramatrical hyphae is supported by the suggestion that the fungi are able to penetrate volumes of soil which are unavailable to the plant roots. It is therefore plausible that the hyphae experience different conditions of water availability than the plant roots, and that they

could be capable of signalling this difference to the host plant. A signalling response of hyphae to water availability, rather than a direct transport of water, may be a mechanism for altered drought response in host plants.

In split-pot studies the proportion of the root system experiencing conditions of adequate or reduced water is varied and the whole plant response examined. Part of the root system is then removed to assess whether adequate water to supply the plant requirement can be obtained from a portion of the root system. This could be repeated with the external hyphae of mycorrhiza to test whether they are also involved in a signalling of drought conditions to the plant, rather than transporting water directly. It would be necessary allow the hyphae to grow into a separate soil volume from the host roots. This has been achieved with the use of rhizoboxes. The external hyphal length of the host-fungus association would be altered by severing the hyphae in this separate soil volume, and monitoring the plant response with and without access to some of the external hyphae.

5.1.6 Metabolic activity of the mycorrhizal tissues

The viability of a fungal mycelium has been shown to decline with age (Kough *et al.* 1987). A fungal signalling mechanism in mycorrhizal water relations could be influenced by the metabolic activity of the hyphae. An assessment of viable hyphal length is then necessary. In addition it was intended to limit the vigour of the hyphae with the use of a fungicide, in order to test this treatment on the water relations of the host plant. Fungicides could be used as an alternative method to remove the influence of hyphae. A range of fungicides have been assessed in their effect on AMF in pot and field trials, and in purpose built microcosms (Kling and Jakobsen 1997, Larsen *et al.* 1996). Commercial fungicides do not target AMF. They are not intended to control AMF. Soft rots and some soil fungi are similar to AMF, but do not show a similar response to the same fungicides. Most studies are carried out either to demonstrate no detrimental effect on AMF, or to determine ways of controlling AMF. Fungicides targeting Oomycetes, such as metalaxyl, might be expected to control AMF, but there is little published data on these. Benomyl has been shown in many studies to be useful in AMF control, particularly on *Glomus* species, despite not targeting Oomycetes. In

these previous studies both a reduction in percentage mycorrhizal colonisation within the root, and a reduction in the external mycelium has been shown.

The importance of the external hyphae of mycorrhizal plants in their water relations is examined in two ways in this study. Initially the hyphae are considered as potentially able to transport water. Evidence for the removal of water from a soil volume by hyphae is examined. If hyphae remove water, is it proportional to hyphal length? The plant response to soil drying is then compared without and without access to these hyphae. The functioning of the hyphae is also reduced using a fungicide, to reduced the overall active length of hyphae to which the plant has access.

5.1.7 Mechanisms for mycorrhizal plant water relations

The objectives of the following experiments are to determine how the quantity of external fungal hyphae affects the host plant response to water availability, and how the response changes when the quantity of hyphae is altered. Three experiments are described in this chapter. They are as follows.

A) The water availability in the soil surrounding the external hyphae is varied by applying measured quantities of water. The removal of water from this volume of soil is monitored and compared to the length of external hyphae occupying the soil.

The quantity of external hyphae is varied in two ways. Both treatments result in a reduction in the hyphal system, but by different mechanisms, and so with potentially different effects.

B) A portion of the external hyphae is severed from the root system. The shoot response is compared before and after removal of the hyphae.

C) The hyphae are chemically controlled. Their functioning is reduced using a systemic fungicide. The shoot response is compared before and after fungicide treatment.

There are three potential options for arbuscular mycorrhizae in relation to plant water uptake. These roles are tested in different ways during all three experiments stated above.

i) First there may be no role, and if this is the case, there will be no change in the plant shoot response to treatments imposed on the external hyphae.

ii) Second, there may be direct transport of water from the soil, via hyphae into the plant root. If this is the case, then the hyphae are acting as extensions of the root system and should respond in a similar manner. The first experiment (A) tests the idea that there is a direct transport of water from the soil via the hyphae to the host plant, which is dependent on the surface area of the hyphae, as water absorbing tissues. The quantity of water removed from the soil should be dependent on the length, and hence surface area of the external hyphae.

In the second experiment (B), when the external hyphae are removed, a decrease in the rate of transpiration should be seen, since the total resistance of the root system is increased as a consequence of its reduced surface area. Stomatal conductance initially will remain constant if there is no change in the atmospheric conditions. However as the reduced conductance of water through the plant continues, the leaf water potential will decrease and give rise to a lowered stomatal conductance.

The application of fungicide to the external hyphae in the third experiment (C), tests whether the transport of water is a metabolically active or passive process. If the external hyphae are killed or their functioning impaired by the fungicide, this has consequences for direct water transport if it is a metabolically active process. If the water transport of the hyphae is influenced by fungicide application this may be shown as a decrease in transpiration, if the transport is active, or no change if the transport is passive.

iii) Third, if there is signalling of the soil water potential by hyphae to the plant shoot, the shoot response will be directly related to the soil water potential. In dry soil there are hormonal changes in the root which are communicated to the shoot. Stomatal conductance is decreased due to this signal, before there is a reduction in leaf water potential as a result of reduced water conductivity. Signalling is also

evident where there is variation in soil water availability. Despite lack of water in one area, a plant is able to maintain its stomatal conductance at the same level as in conditions of adequate water availability. In experiment (A) where there is greater water availability in the soil volume surrounding the external hyphae, than surrounding the host roots, stomatal conductance should be maintained if the hyphae are able to signal to the host.

In experiment (B), the stomatal conductance would not be decreased in dry soil conditions when the external hyphae are removed, since the drying signal is not received in the shoot.

Similarly in experiment (C) the application of fungicide could cause stomatal conductance to remain stable in dry soil if the hyphal signalling is inhibited.

5.2 Materials and Methods

These experiments were carried out in a specially designed rhizobox based on one used by Faber *et al.* (1991). This is made up of two sections, one where both roots and associated hyphae are present, and a second where hyphae are able to grow into a volume of soil into which roots have no access. This is in order to simulate the situation in field soil where hyphae are growing into soil pores which are too small for roots to penetrate. This then examines that idea that hyphae are better able to exploit the heterogeneity of soil than a root system with no mycorrhizal colonisation and so improve the ability of the host plant to avoid conditions of low water availability.

Materials

5.2.1 Soil preparation

Soil from the Aldroughty area was used for this work, because of its low organic matter content, to maximise the growth of arbuscular mycorrhizal fungi. It has been shown that the colonisation and growth of AMF is reduced by high concentrations of nutrients, particularly phosphate (Amijee *et al.* 1993, Bruce *et al.*, 1994). The soil was sieved to 4 mm and steam sterilised twice at 121^{O}C and 100 kPa for 1 hr in an autoclave (Midas 32, Priorclave). It was packed to a bulk density of 0.64 g cm^{-3}, calculated from the dry weight of soil and the volume of the rhizobox. The cuttings described in Chapter 3 were miniaturised, growing to no more than 25cm. The bulk density of soil in this experiment was therefore reduced to 0.64g cm^{-3} in order to avoid any root constriction which was a potential cause of this. In addition to its low phosphate concentration the Aldroughty soil was chosen because of its low clay content and less dense structure (see Appendix 1). Cuttings described in this section grew to be over twice the height of those in Chapter 3. The soil water potential of Aldroughty soil was calculated from the equation derived by Bache *et al.* (1981) for Corby Series which is the major soil group in the area around Elgin from which Aldroughty soil is collected.

The gravimetric soil moisture content derived from the resistance cells was converted to volumetric soil moisture content using

$$\theta v = \theta g \times \rho$$

where θv = volumetric moisture content

θg = gravimetric moisture content

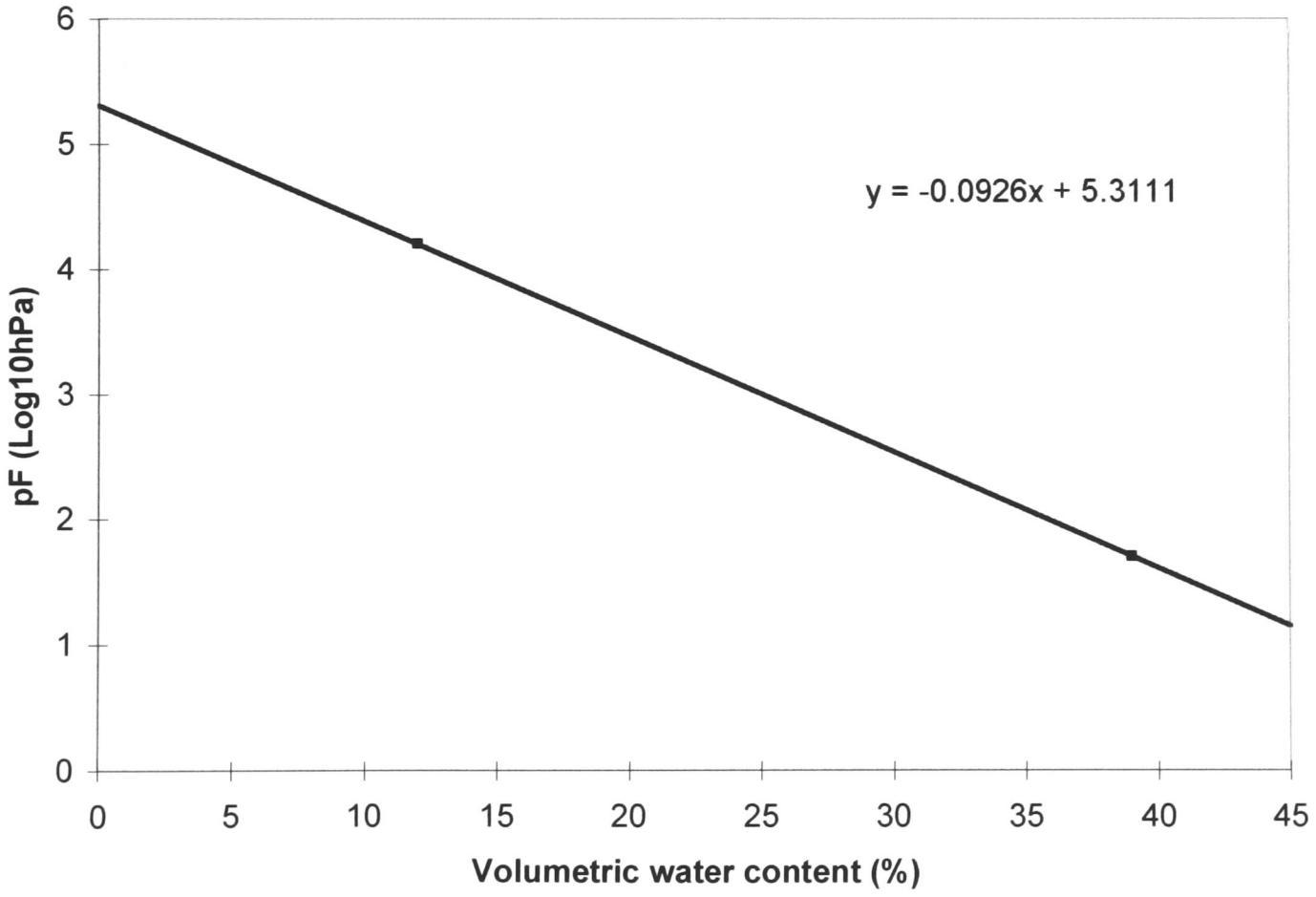
ρ = soil bulk density

The equation from by Bache *et al.* (1981) for Corby Series soil moisture release curve is

$$pF = -0.0926 \times \theta v + 5.31$$

This is shown below in Figure 5.1. At field capacity, when soil water potential is taken as -0.005MPa, and pF = 1.7, volumetric soil moisture content is 39%. At permanent wilting point when soil water potential is -1.5MPa, and pF = 4.2, volumetric soil moisture content is 12%.

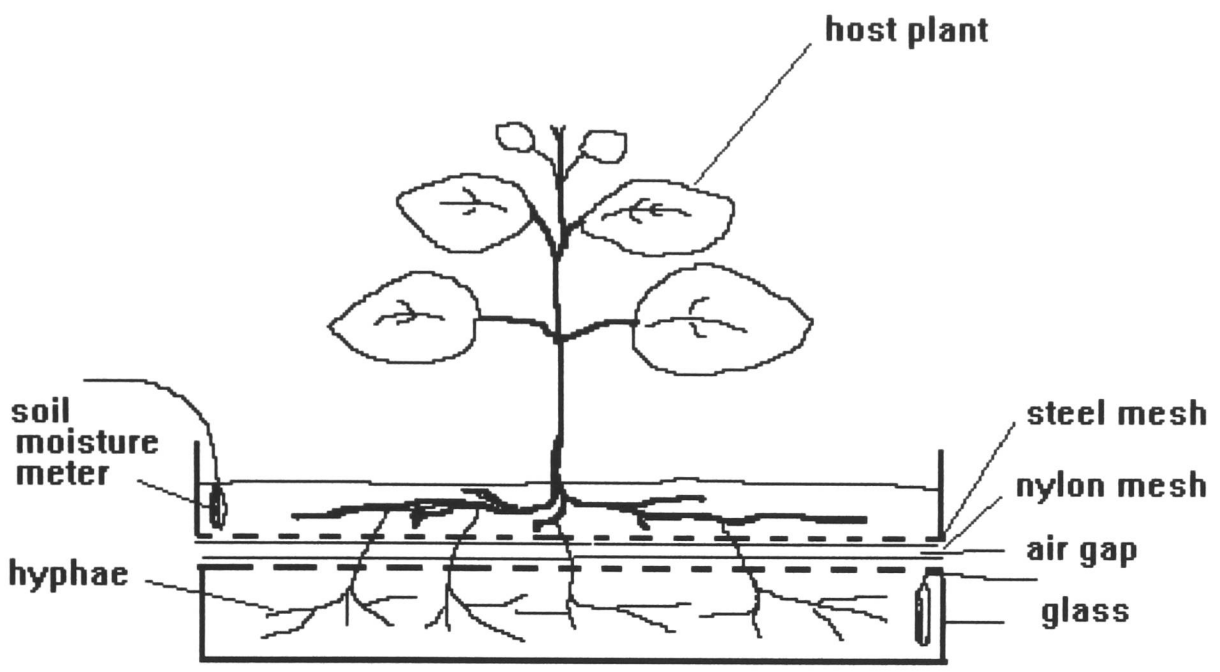
Fig.5.1 Soil moisture release for Corby Series soil (Bache *et al.* 1981)



5.2.2 Construction of the rhizobox

The rhizobox was made up from a flat-bottomed dish (Duran crystallising dish, Norlab Glassblowing Services Ltd. (Fig.5.2). It was 18 cm diameter and 10 cm high. The base was cut off at a height of 2 cm to give two sections which fitted together. Between these two sections two dividers were glued with silicone sealant (Silastic Adhesive Sealant Rubber for Corrosion-Sensitive Components, BDH Merck Ltd.). A divider was made up of a circular disc of perforated steel sheet, 0.7 mm thick, with hole diameters of 1.2 mm (RS Mechanical Products and Tools, UK), with nylon mesh of pore size 30 μm (Plastok, UK) glued to it. The steel sheet was used as a stronger barrier to root growth than the nylon mesh. One divider was glued on to the base of the upper section, with the steel side uppermost, and another was glued onto the lower section with the mesh side uppermost, to prevent roots from entering the lower section. Soil and a resistance cell (Soil moisture and temperature cell, ELE International Ltd.) were put into the lower section before sealing it. A 1.5 mm bore plastic straw (Mastercalf) was also inserted into the lower section so that one end rested in the centre of the dish and its opposite protruded approximately 2 cm. This straw was used to water the hyphal section once it was completely sealed, by inserting a syringe into the protruding end and injecting the required amount of deionised water. The upper and lower sections were glued together with 2 mm spacers between them to give an air gap. This was to prevent the transfer of water vapour between the sections. This was tested by wetting the soil in the lower section to the wettest extreme measurable using the resistance cell, which was equivalent to 60% gravimetric water content. The resistance was monitored at temperatures of 10-20^{O}C under glasshouse conditions. There was no measurable loss of water from the lower section. From these results, it was deduced that evaporative losses from the lower section were negligible. The volume of the upper section was 1655 cm^3 and that of the lower section 345 cm^3. The volume of the lower section was deliberately kept small so that small changes in the quantity of water in the soil could be detected. The weight of dry soil in the upper section was 1052 g and in the lower sections was 219 g. Twenty rhizoboxes were made and used.

Fig.5.2 Construction of the rhizobox



5.2.3 Growing conditions for rhizobox experiments

Plants were maintained in rhizoboxes for two growing seasons as described in Chapter 2, so that during this time the quantity of hyphae in the hyphal section could increase. Data was collected during this period on the soil water content of each section, and on the gas exchange parameters of each plant at the same time.

Each rhizobox was planted with a cutting, with 4-5 buds, derived from 1 year whips of hybrid black poplar cv. Robusta. A hole was made in the soil to approximately 3 cm depth. Chopped roots (0.2 g) from onion stock plants colonised by *Glomus intraradices* FL-208-4 was used as a source of inoculum. This was put into the hole with the cutting above it. It ensured that the young roots of the cutting came quickly into contact with the inoculum. Nineteen rhizoboxes were inoculated and planted. The twentieth was left unplanted. It was to be used to measure the quantity of hyphae to be found in the uninoculated soil, i.e. the background level of hyphae of all species, not just the chosen mycorrhizal species.

After 8 weeks root and soil samples were taken from the upper section using a 1cm diameter steel borer. At this stage there was no evidence of mycorrhizal

colonisation. Sampling was continued at intervals of 4 weeks. Measurements were begun 4 months after planting and inoculation. The plants were maintained under glasshouse conditions for 1 year. Air temperatures ranged from 10 to 30^{O}C. Light intensity, under sodium lamps (SontAgro), was 100 to 900 μmol $m^{-2}s^{-1}$, depending on the time of year. They were watered with deionised water (Millipore), according to requirement, when the soil dried to 25%. The plants were also supplied with 1/2 strength Hoaglands nutrient solution (see Appendix 2) approximately monthly. The application of nutrient solution was restricted to promote growth of mycorrhizal hyphae.

After the first season 8 plants were harvested. The remaining rhizoboxes were put outside on a gravel bed to chill them so that a natural rhythm of dormancy in the poplar would be achieved and broken. The soil temperature dropped from 30 to 15^{O}C. Due to mild winter temperatures, it was doubtful as to whether the saplings would experience enough chilling to ensure dormancy. They were therefore put into cold store at 5^{O}C for three weeks, and then returned to the gravel bed. Three plants died during this time.

After 4 months outside the shoots came into leaf again. They were put back into the glasshouse. Further experiments were carried out, in the second season.

Initially there were 19 inoculated rhizoboxes plus one uninoculated to provide a base level of hyphae in the soil in the hyphal section. The plant root section was deliberately limited to maintain conditions of restricted rooting, so that the host plant would benefit from any potential access to soil water in the hyphal section. However this made it difficult to maintain all the plants in a healthy state for two seasons, and half were harvested after the first growing season. Following the winter the two subsequent experiments were continued with greatly reduced replicate numbers. The following analyses were carried out on the first season data initially, and then repeated during the two subsequent experiments. The reduced number of replicates clearly influenced the sensitivity of the experimental analyses.

Methods

Three major studies were carried out. The first involved monitoring the reduction in soil moisture content in the hyphal section of the rhizobox, during drying cycles. The second involved a comparison between the leaf water loss and metabolic processes and soil drying, before and after the hyphae were separated from their host plant. The third study concerned the contribution of live or dead hyphae to water uptake. The measurements taken during these three experiments are described below, and followed by details of the methods used in each of the experiments.

Measurements

5.2.4 Soil water uptake from the root+hyphal and hyphal sections of the rhizobox.

The rhizobox was made up of a section containing roots and associated hyphae, and another section containing only hyphae. The changes in water uptake from each section of the rhizobox were measured, using the moisture resistance units inserted into each sections of the rhizobox. The measured resistance was converted to a gravimetric soil moisture content as described in Chapter 2 and used to calculate the soil water potential and the quantity of water removed from the soil.

5.2.5 Plant and fungal characteristics

The shoot weight, root weight, leaf area and percentage mycorrhizal colonisation were measured at harvest using the methods described in Chapter 2, and are presented in Tables 5.2 and 5.3.

5.2.6 Determining the extent of the hyphal system

The quantity of hyphae in the hyphal section of the rhizobox was measured. A method adapted from Miller and Jastrow (1992) was used to remove mycorrhizal hyphae from the soil so that the length of hyphae in the hyphal section of the rhizobox could be determined. The water content of the soil was found by weighing a quantity of soil, drying it overnight at $80^{O}C$ and reweighing. A sample of soil of approximately 5g dry weight was taken from the unsealed mycorrhizal section, and weighed

accurately to 0.1 g. It was mixed with 250 ml deionised water in a flask and thoroughly stirred until the large aggregates were broken up. This was the first dilution of the soil sample. The flask was then returned to the automatic stirrer and maintained at a speed so that the bottom of the vortex was halfway between the surface of the solution and the base of the flask. This allowed larger aggregates to remain on the flask bottom but allowed hyphae to spin up. Two samples of 6 ml were removed from the first dilution with an automatic pipette filler (Gilman), from the same position in the flask, and put into a fresh flask. The sample was diluted with 125 ml deionised water and 15 ml of the dispersant 3.5% w/v sodium metahexaphosphate. The dispersant was necessary to break up soil colloids. The filtrate was allowed to settle for 20 minutes. It was then returned to the automatic stirrer and run at a low speed to resuspend hyphae and fine colloids. Two samples of 10 ml were removed successively from the same position in the flask. This 20 ml sample was used for assessing hyphal density. It was filtered through a 210 μm sieve, which effectively removed pieces of organic matter. The filtered solution with rinsings was put into a flask on a vacuum pump. In order to test whether the 210 μm sieve was removing only soil organic matter, the material remaining on the sieve was also washed into the vacuum flask and collected on a fresh glass fibre filter paper. Microscopic examination indicated that only a few hyphae remained on the sieve surface.

The filtrate with hyphae was filtered onto a nylon mesh filter of pore size 20 μm. This would allow fine colloids to pass but retain hyphae. The filtrate was collected onto a glass fibre filter paper and examined microscopically. No hyphae were found, indicating that no hyphae passed through the 20 μm mesh filter. The mesh was removed carefully and allowed to stain overnight in 5 ml 0.05%w/v trypan blue in lactoglycerol. After 24 hours the stain and rinsings from the mesh were put back into the vacuum pump flask and the solution extracted onto a glass fibre filter disc of pore size 1.2 μm (Whatman). This disc was examined microscopically to measure hyphal density. Six discs each from 20 ml samples of the second dilution were made up and examined to give a mean value for hyphal density.

A dissecting microscope (Carl Zeiss Jena, Glasgow) at magnification x30 was used with a grid in the eyepiece. The method of Newman (1966) was used to

calculate the length of hyphae from the number of intersections between a line on the grid and a hyphal fragment.

The relationship is given as;

$$R = \frac{\pi A N}{2H}$$

where R = total length of hyphae
A = area of the field
N = number of intersections between hyphae and gridlines
H = total length of gridlines

5.2.7 Measurement of live hyphae

Trypan blue is used as a stain for both live and dead fungi. However it was also necessary to estimate the quantity of live hyphae in the soil. For this a vital stain was required. Tetrazolium red (2,3,5-Triphenyltetrazolium chloride) was used. It has been widely used to assess the viability of seeds and spores. It gives a clear colour change in live tissue. Unlike a fluorescent stain it can be seen under the dissecting microscope and so the previous procedure needed only slight modification.

The procedure adopted for live hyphae was similar to that used for the total hyphal count. The first dilution was obtained in the same way. In the second dilution only 113 ml of deionised water was added, so as to reduce the extent of the dilution. Sodium metahexaphosphate was excluded, in order to avoid a potential effect on the vitality of the hyphae. The final volume of the second dilution was 125 ml. Without the dispersant, the colloids in this solution were larger. The samples from this second dilution were filtered through a 250 μm sieve. At all times pre-chilled deionised water, kept refrigerated at 5^{O}C, was used to make solutions so as to maintain the vitality of the hyphae. The 20 μm nylon filter was stained in 4 ml 1%w/v tetrazolium red made up in deionised water. Staining periods of 15, and 30 minutes, and of 5 and 20 hours were assessed. It was found that staining was most consistent at 20 hours, in that the most hyphae were visible and remained visible after reassessment 1 hour later. The nylon filter was therefore kept overnight in the stain, at 20^{O}C in the dark, in an incubator. This allowed the hyphae to respire. Equivalent samples were also stained in 4 ml 5% trypan blue in lactoglycerol. Five replicate glass fibre discs were made up for each of the stains. The discs were examined microscopically as above, at x24

magnification. It was found that hyphae treated with tetrazolium red could be unstained and clear, or pink, or blue in colour. This variation is discussed further in section 5.7. Both blue and pink hyphae were taken as respiring therefore alive.

5.2.8 Plant shoot response to changes in soil water around roots and hyphae

The leaf processes transpiration and photosynthesis, and the leaf stomatal conductance were derived from measurements using an IRGA as described in Chapter 2. These were compared to water availability in the soil sections, occupied by the roots and by the hyphae. Stomatal conductance and transpiration rate were used to assess the loss of water from the leaves. Photosynthetic rate was used as a measure of the plant metabolism. These leaf processes of plants when grown in rhizoboxes were examined to assess whether their response to environmental conditions was altered by growth in a rhizobox. They were then monitored for any change when the hyphal section was treated in one of two ways in Experiments (B) and (C) described below. These were severing of hyphae from the root section, or chemical control of the hyphae using benomyl fungicide.

5.2.9 Description of experiment methods

Experiment (A): To monitor the extraction of water by hyphae

The plants were subjected to a series of drying cycles throughout the year. The plants were watered in the upper plant section with a measured volume (ml) of deionised water to give a water content of approximately 45%, as calculated from the measured resistance of the moisture cell. The water content in the lower section of the rhizobox was adjusted to 55%. The rhizoboxes were then left to dry for the number of days required to reach a gravimetric water content of 25% in the upper section. This usually took 3-4 days during the summer. The water contents in the upper and lower sections were monitored daily during this time. Occasionally 1 or 2 plants began to wilt and were clearly experiencing greater water deficit stress than the others. Supplementary water was supplied to these plants. However this was largely avoided, and in general all replicate plants were maintained at similar water contents. Once the water content in the lower section had reached 48% it was watered via the watering tube with a known quantity (cm^3) of deionised water. The lower section was generally wetter than the upper, and so acted as a potential source of water.

After 12 months, 8 plants were harvested. The upper section of the rhizobox was dismantled and the plant removed. The shoot was removed from the original cutting and weighed. The soil, with the root system, was put into a bucket with 10 litres 0.05% w/v sodium hexametaphosphate, for 48 hours agitating periodically, to remove some soil. The roots were washed further under running water, using a 1 mm sieve to collect root fragments. The fresh and dry weights of the root system were found. The lower section was opened by passing a spatula between the steel sheet and the glass base, see Fig.5.2. The soil was removed and put into a plastic bag and stored at $5^{O}C$. This sample was used for the assessments of hyphal length which is described above. Water use was correlated with the measured hyphal lengths in order to obtain an estimate of the quantity of water which could be transported by the hyphae.

Experiment (B): To determine the effect on the host plant of removal of associated mycorrhizal fungi.

This experiment was designed to study the effect of removing the extramatrical hyphae from contributing to water transport. This was achieved by passing a large knife through the air gap in the microcosm. In this way the immediate effect on the water relations of the host plant, and any continuing uptake of water from the hyphal section, which could not be due to hyphal activity, could be monitored.

The soil water content was measured before the experiment started, using the resistance cell in the lower hyphal section of the rhizobox. The youngest fully expanded leaf for gas measurement in a IRGA leaf chamber, was selected. In this way a vigorous, actively photosynthesising leaf with a large surface area, was used for the measurement of gas exchange. The leaf was fitted into the leaf chamber which was then held in position with a clamp stand. The IRGA was programmed to take readings, automatically, every 2 minutes. This was continued for 10 minutes. At this stage the top and bottom sections of the rhizobox were severed with a knife, while the IRGA remained connected to the leaf. Readings using the IRGA were taken for a further 10 minutes. The leaf chamber was then removed. The IRGA screen showed that the temperature of the leaf gradually increased while held in the chamber. The cut surfaces of the two sections around the air gap, were covered immediately with polyethylene film to prevent water loss from those surfaces. After 1 hour leaf gas exchange was remeasured. The soil water content in the lower chamber was again measured after 48 hours to assess continued loss of soil moisture, by non-hyphal routes.

The lower section was opened, the soil removed and put into a plastic bag and refrigerated at $5^{O}C$ to maintain the vitality of any fungal hyphae present. This was used for an assessment of hyphal length as described above.

The upper section of the rhizobox was dismantled and the plant harvested. The leaves were collected into a plastic bag. The fresh weight was found to an accuracy of 0.001 g, and the dry weight after drying at $80^{O}C$ overnight. Before drying the leaf area was measured using an image analysis system (Quantimet 600, Leica

Cambridge Ltd.). The shoot was removed from the original cutting and weighed to 0.1 g. The soil with the root system was put into a bucket with 10 litres 0.05% w/v sodium hexametaphosphate, for 48 hours agitating periodically, to remove some soil. The roots were washed further under running water, using a 1 mm sieve to collect root fragments. The fresh and dry weights of the root system were also found.

Experiment (C): To determine the relative importance of active and inactive hyphae.

An experiment was carried out to determine whether any water transport carried out within the hyphae is due to metabolically active or inactive hyphae. A review of commonly available fungicides was carried out, the target fungi against which they are used, and their effects on arbuscular mycorrhiza. Table 5.1 summarises the effects on the fungal and plant partners of various crop protection chemicals. They are discussed in further detail below.

Table 5.1 Effect of plant protection chemicals on AMF and host plants

Fungicide	AMF colonisation	Host	Fungus	Reference
Benomyl	↓	Pea	mixed	Fitter&Nichols 1988
	↓	Clover		
Benomyl	↓	Onion	*Glomus*	Sukarno *et al.* 1993
Benomyl	↓	Barley		Spokes *et al.* 1981
Captan	↓	Onion	*G.intraradices* *G.caledonium*	Kough *et al.* 1987
Drazoxolon	↓	Onion		Spokes *et al.* 1981
	↓	Lettuce		
Triadimefon	↓	Onion		Spokes *et al.* 1981
	↑	Lettuce		
Etridiazole	↓			Spokes *et al.* 1981
Chloroneb	↑		*G.microcarpus*	Spokes *et al.* 1981
Metalaxyl	↓	Leek	*G.intraradices*	Jabaji-Hare&Kendrik 1987
Fosetyl-Al	↑	Leek	*G.intraradices*	Jabaji-Hare&Kendrik 1987
Fosetyl-Al	--	Pineapple	*G.aggregatum*	Aziz *et al.* 1990
PCNB	↓	Oats	*G.mosseae*	Gnekow&Marschner 1989
Carbendazim	↓	Pea	*G.intraradices* *G.clairoideum* *G.invermaium*	Kling & Jakobsen 1997
Propiconzole	--	Pea	*G.intraradices* *G.clairoideum* *G.invermaium*	Kling & Jakobsen 1997

The effect on the host plant of application of the fungicide was also important as a consideration. Benomyl had no effect on plant growth (shoot dry weight or root length) but had a direct effect on all aspects of growth of the fungus. It reduced living internal hyphae, arbuscles, and living external hyphae.

Fosetyl-Al reduced root growth. Generally it had no affect on the amount or characteristics of fungal colonisation but in some cases it increased colonisation, possibly due to an increase in root exudates translocated in the transpiration stream.

There was no effect on living intercellular hyphae and arbuscles. It reduced the length of infected root per plant and the area of interface between plant and fungus. External hyphal length was also reduced but less than with benomyl.

Metalaxyl application reduced plant growth and number of living intercellular hyphae, arbuscles, fungal-plant interface, length of infected root and development of external hyphae. Metalaxyl acted by reducing the amount of fungal colonisation per plant, rather than affecting the function of the fungus in the root.

Pentachloronitrobenzene (PCNB) had a phytotoxic effect. Total P uptake decreased with increasing PCNB and was correlated with infected root length.

Captan appeared to have a phytotoxic effect.

Carbendazim (Bavistin) inhibited P-32 transport and succinate dehydrogenase activity in external mycelium.

Propiconazole (Tilt) had no effect on either mycorrhizal colonisation or function.

The effect of fungicides may be dependent on the particular plant-fungal association, concentration and method of fungicide application and host growth conditions. Benomyl was the most inhibitory chemical. It was selected to reduce the length of active hyphae in the lower section of the rhizobox. Benomyl is a systemic carbamate fungicide with protectant and eradicant activity (The UK Pesticide Guide 1997).

Fungicide application

The dosage of benomyl fungicide applied in this experiment was similar to that in other studies (Merryweather and Fitter 1996, Fitter and Nichols 1988, Larsen *et al.* 1996, Spokes *et al.* 1981, Kough *et al.* 1987, Habte 1997). These are generally greater than the recommended field rates of application. Benomyl is effective in fungal control whether incorporated in soil before planting or sprayed onto soil as a suspension (Fitter and Nichols 1988).

The water content of the upper and lower sections of the rhizobox was measured at the start of the experiment using the resistance sensors in each section. The gas exchange was measured on four fully expanded vigorous leaves from different heights on the plant. A benomyl suspension (3 g l^{-1}) was made up and 5 ml

injected into the hyphal section of the each rhizobox. The water content in the hyphal section and the gas exchange of the plants were recorded for a further 9 days. The plants were then harvested. The shoots and roots were separated and weighed, providing fresh and dry weights to 0.1 g. The fresh and dry weight (to 0.001 g) and area (mm^2) of the leaves were also found. The soil from the hyphal section was removed as described in the previous experiment and used for the assessment of hyphal length.

5.3 Results

5.3.1 Measurements of plant characteristics

The measurements recorded on root and shoot weights, and leaf areas, are given in Table 5.2. There is high variation in all parameters measured. This data is used in further analyses. During the first season data was taken from all the plants listed below. During the second season, two further experiments were carried out using plants 12-15 for hyphal severing and plants 16-19 for chemical removal of hyphae. The response of plants 12-19 during these two second experiments is compared to that of the first season. Plants were selected for experimentation in the second season on the basis of their observed vigour, and on their similarity in shoot height and leaf area. It can be seen that plants 12-15 have similar leaf areas. Plants 16-19 show greater variation in leaf area but similar shoot weights. Those plants which were considered less vigorous as defined by their shoot growth, were harvested after one season. Plants 9-11 were not used in further experimentation during the second season due to their observed low vigour. It can be seen that except for plant 10, this is demonstrated in their low root and shoot weights. Leaf area was measured only on those plants used in experiments on hyphal severing and chemical removal, where detailed analyses of leaf gas exchange were carried out.

Table 5.2 Summary of plant measurements

Plants grown for one season

Plant number	Fresh wt roots (g)	Dry wt roots (g)	Fresh wt shoots (g)	Dry wt shoots (g)
1	89.5	23.9	11.8	10.1
2	94.8	22.6	16.2	13.8
3	58.2	13.1	10.2	8.9
4	64.0	16.4	11.7	10.2
5	12.2	2.6	4.4	3.8
6	49.9	8.4	8.8	7.7
7	14.2	2.2	5.1	4.7
8	76.2	16.8	12.1	10.0

Plants grown for two seasons

9	19.9	3.7	6.4	5.2
10	37.1	5.9	13.2	7.2
11	19.1	3.2	7.0	5.1

Plants used for hyphal severing experiment

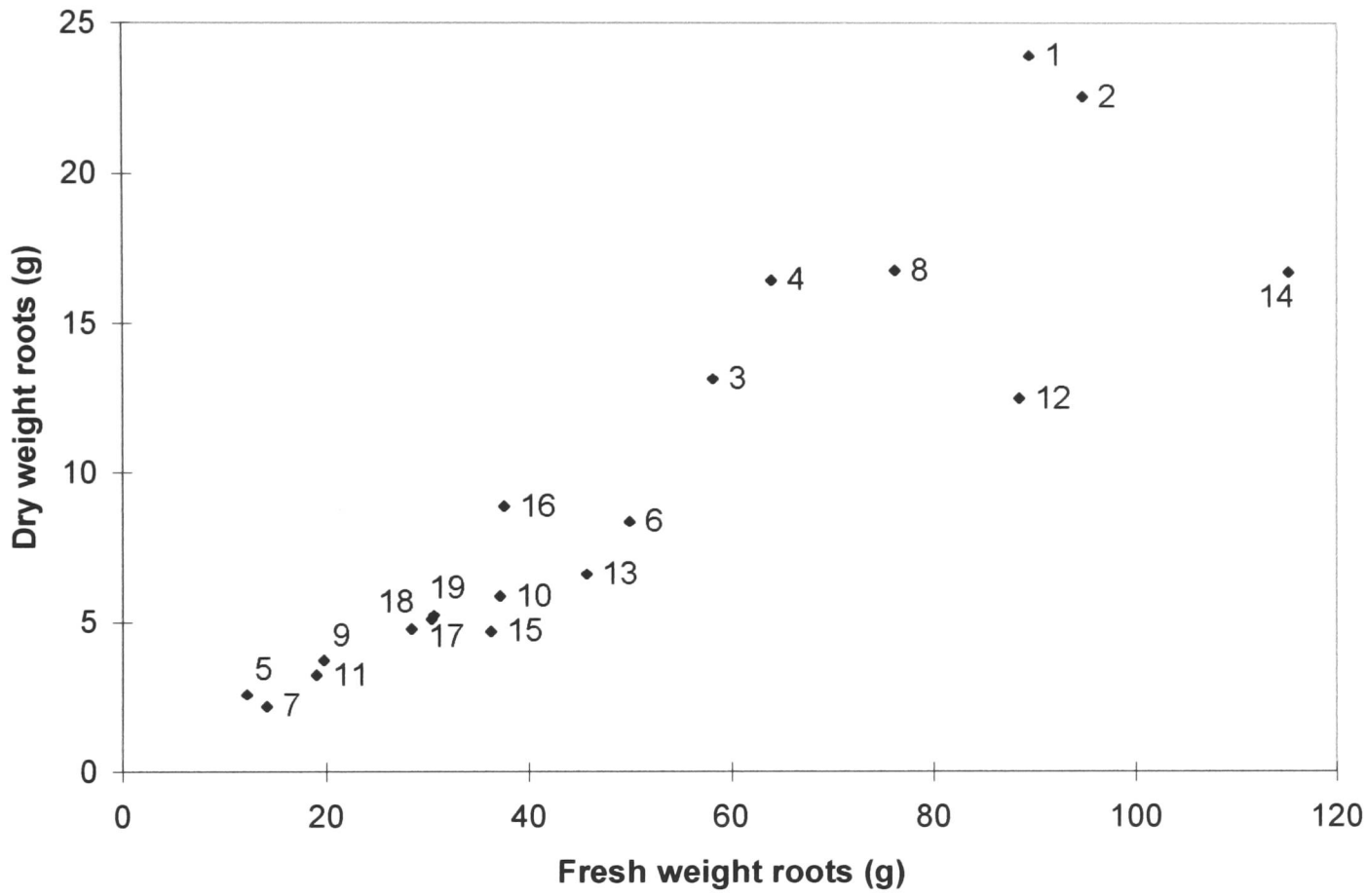
Plant number	Fresh wt roots (g)	Dry wt roots (g)	Fresh wt shoots (g)	Dry wt shoots (g)	Leaf area (m^2)
12	88.5	12.5	31.4	13.0	3.22
13	45.7	6.6	19.6	8.3	2.99
14	115.2	16.7	36.1	18.5	3.26
15	36.3	4.7	18.9	8.6	3.45

Plants used for fungicide control experiment

16	37.5	8.9	11.3	6.6	1.55
17	30.3	5.1	10.4	4.9	0.79
18	28.4	4.8	13.4	6.2	0.97
19	30.6	5.2	13.0	6.5	0.45

In order to check the accurate measurement of samples after harvesting, the fresh and dry weights of the root system and shoots were compared. Figure 5.3 shows the fresh and dry weights of the root system. There is a close relationship between the sample weights at low values. However heavier samples showed greater divergence between fresh and dry weights.

Fig.5.3 Root system fresh weight and dry weight of samples at harvest. Plant numbers are indicated.

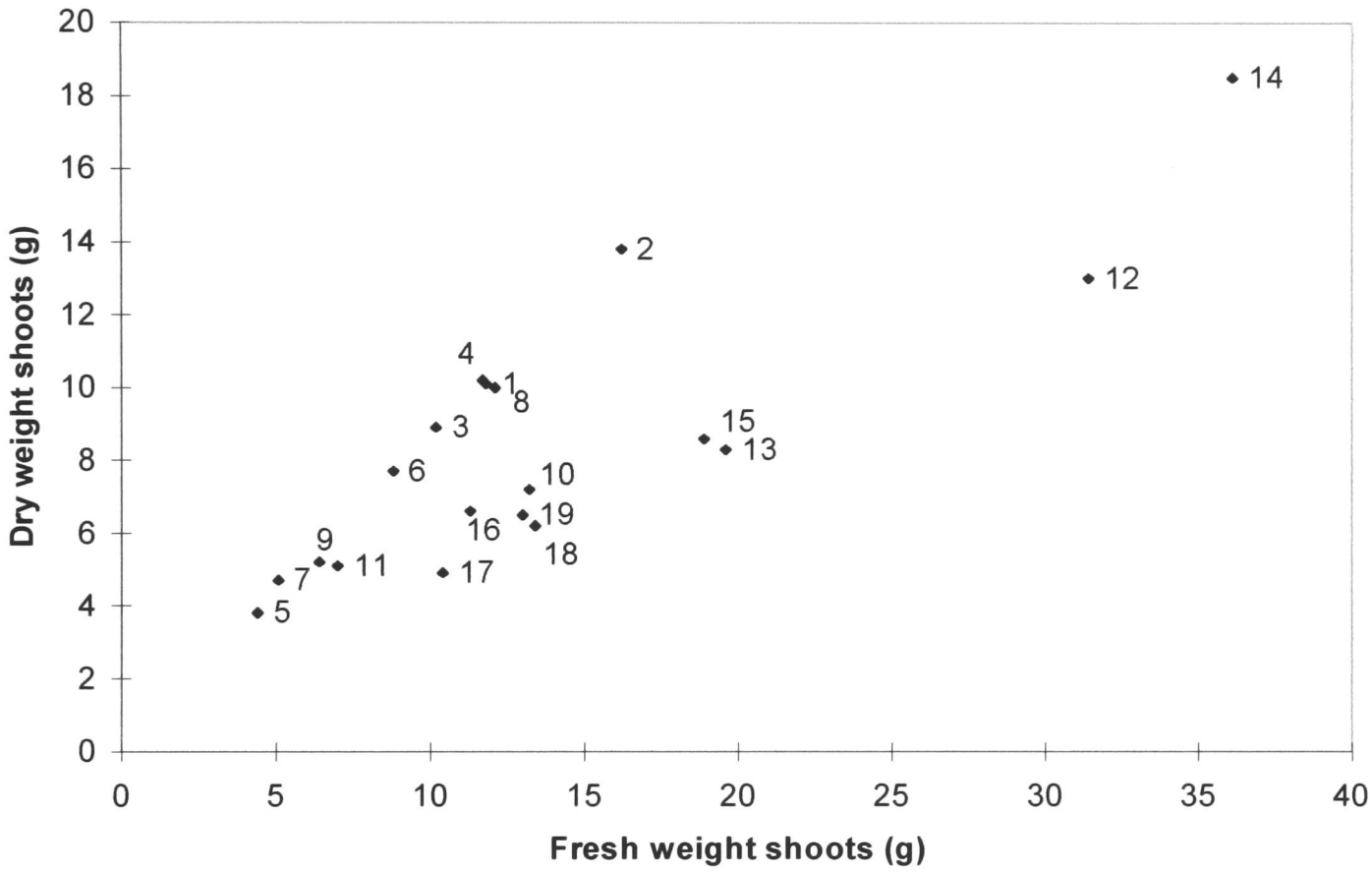


The root system of a plant harvested from a rhizobox is shown in Fig.5.4. The main lateral roots can be seen. They extended to the sides of the rhizobox. Narrower roots formed a network filling the rest of the soil volume in the rhizobox section.

Fig.5.4 Root system of poplar plant in rhizobox

Figure 5.5 shows the shoot fresh and dry weights of the shoots at harvest. The data appear to follow two relationships between the fresh and dry weights. This effect may have been due to the variation in form of the shoots. Cuttings were seen to develop a single stem only, or up to three lateral shoots. Due to the resulting differences in thickening, this may have caused the differences in dry weight.

Fig.5.5 Shoot fresh weight and dry weight of samples at harvest. Plant numbers are indicated.



5.3.2 Mycorrhizal colonisation

Percentage mycorrhizal colonisation and length of external hyphal in the hyphal section of the rhizobox was measured and the results are presented in Table 5.3 below. Percentage mycorrhizal colonisation varied widely between plants, from 18-54%. There was no clear difference in percentage colonisation between plant groups. The lowest percentage mycorrhizal colonisation was seen in plant 9 which was among those rejected in the second season due to low vigour. The greatest colonisation was seen in plants from the first group, harvested after one season. The length of hyphae in the soil collected from the hyphal section of the rhizobox is given in three forms. The total length of all hyphae found is given as "Total length AM and soil hyphae". Given that AM hyphae cannot be distinguished from free-living soil fungal hyphae, a measure of AM fungal length only is given as "Length AM hyphae – soil hyphae" by removing the length of hyphae found in a control uninoculated rhizobox. As AM fungal hyphae may reduce the populations of free-living fungi in soil, either measure may be appropriate in comparisons of hyphal length between the sampled plants. The final measure is of actively metabolising hyphae stained in tetrazolium blue, referred to as "length viable hyphae". There is great variation in the length of hyphae between sampled plants. The greatest length was found in plant 14 in the group used for hyphae severing experimentation. No evidence of AM fungi was seen in plants 4,6, and 10.

Table 5.3 Summary of mycorrhiza measurements

Plants grown for one season

Plant	Mycorrhiza	Total length AM & soil hyphae (mm/g)	Length AM hyphae – soil hyphae (mm/g)
1	52%	1139.4	558.4
2	47%	1609.8	1028.8
3	42%	680.8	99.8
4	47%	349.7	0.0
5	40%	1346.5	765.5
6	54%	158.3	0.0
7	25%	1717.1	1136.1
8	33%	996.1	415.1

Plants grown for two seasons

Plant	Mycorrhiza	Total hyphal length (mm/g)	Hyphae - control length (mm/g)	Length viable hyphae (mm/g)
9	18%	683.4	102.4	448.0
10	42%	516.0	0.0	95.0
11	27%	602.3	21.3	166.5

Plants used for hyphal severing experiment

12	50%	779.8	198.8	188.1
13	36%	1340.0	759.0	173.4
14	26%	4949.0	4368.0	87.9
15	41%	867.7	286.7	128.1

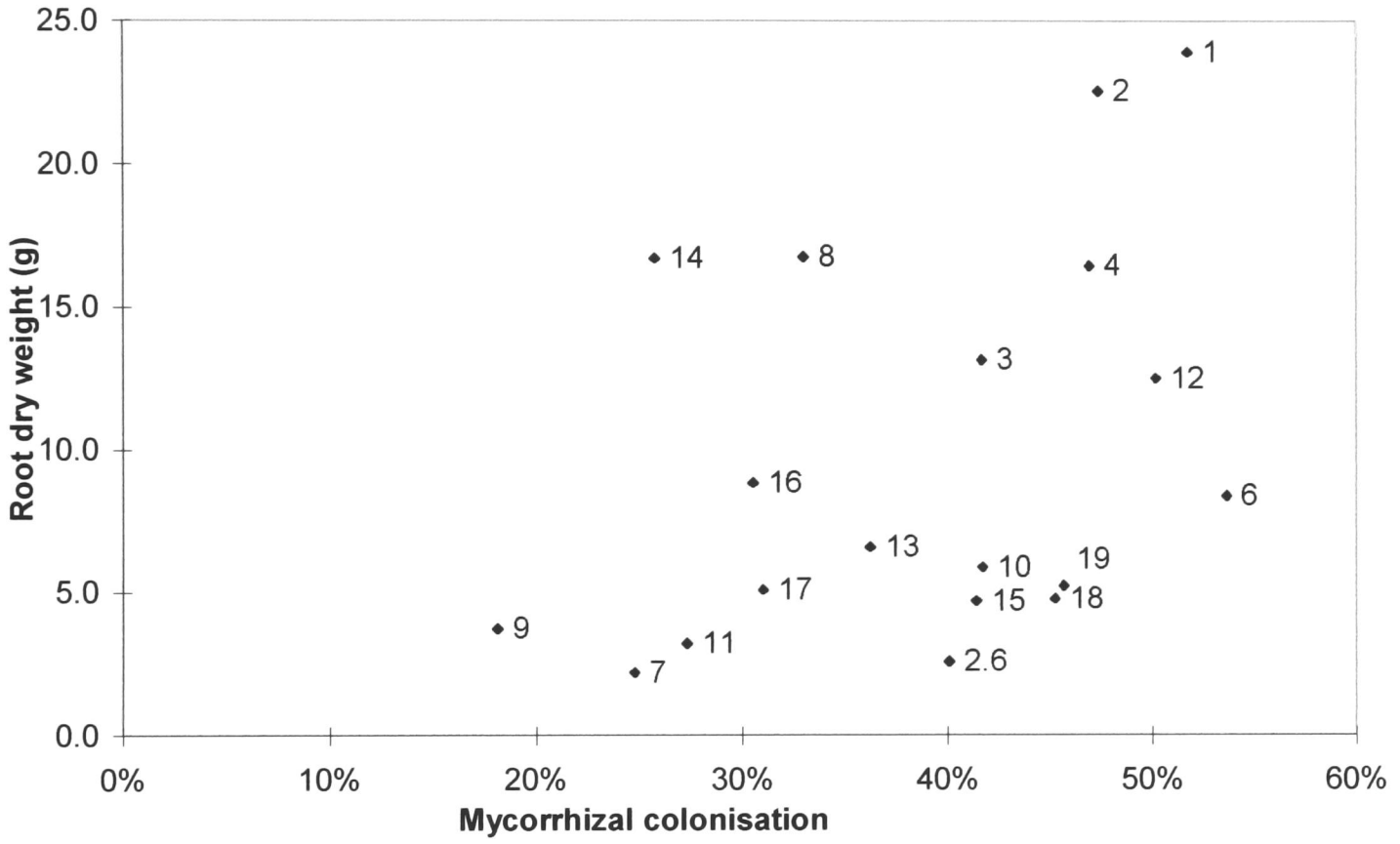
Plants used for chemical reduction experiment

16	31%	690.2	109.2	197.5
17	31%	1173.0	592.0	215.6
18	45%	1258.6	677.6	93.3
19	46%	1140.2	559.2	289.4

The percentage mycorrhizal colonisation and length of external hyphae were compared to the mass of the plant shoot and roots. This was in order to assess whether differences in the fungal partner in the symbiosis were strongly related to the observed variations in plant characteristics. The correlation coefficient "r" is given in Table 5.4. These values give the likelihood of a linear relationship between each tested parameter. However it can be seen in the following scatter graphs that the relationships were not always linear. Percentage mycorrhizal colonisation was

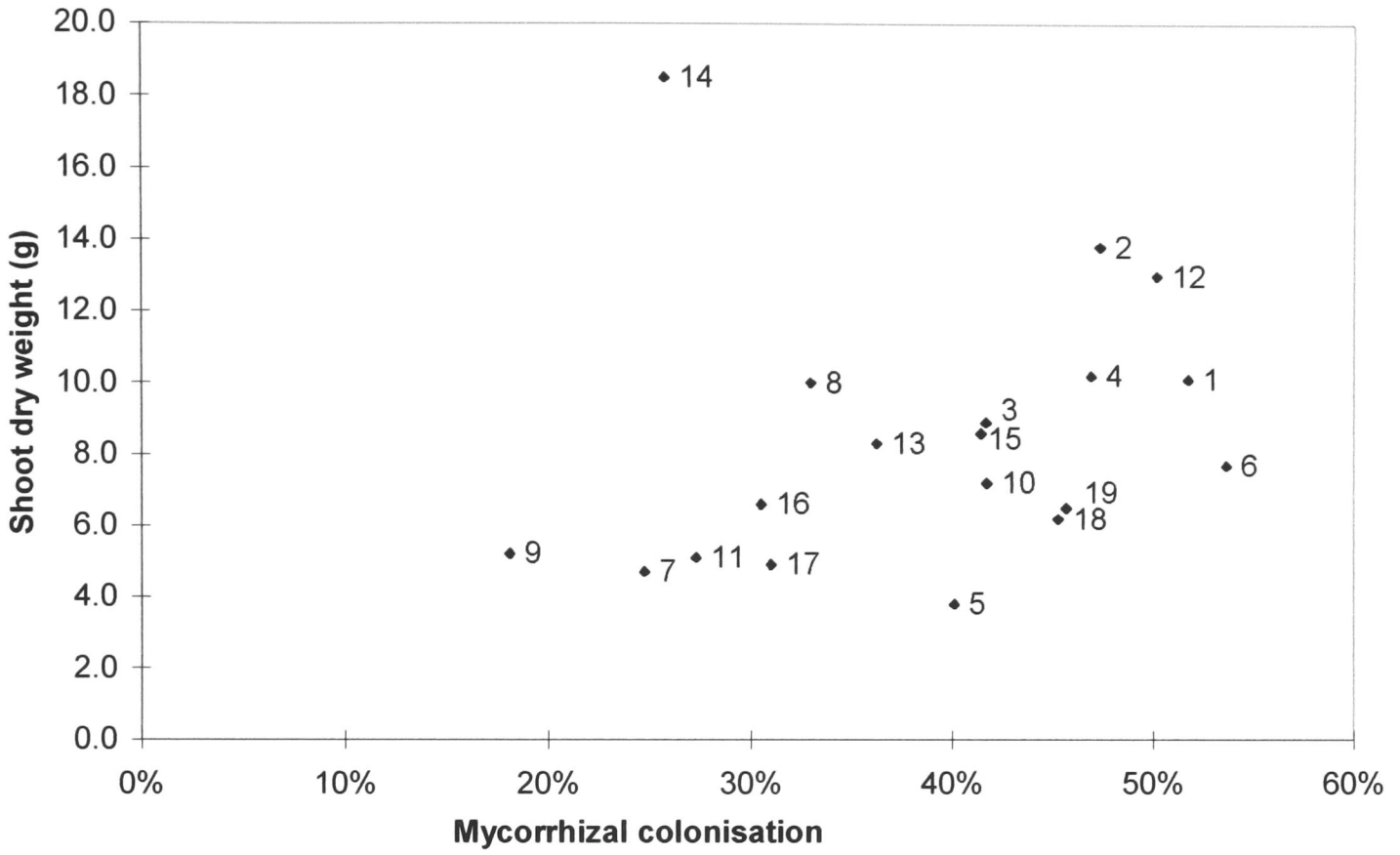
positively related to root and shoot dry weight (Figs.5.6,5.7), although these correlations were not statistically significant. There was no clear relationship between the root dry weight at harvest and the age of the plants, but those grown for only one season appeared to show a greater increase in root dry weight with greater mycorrhizal colonisation, than those grown for two seasons. Those plants which survived until the second season appeared to be those with lower root weight, possibly because they were not yet suffering root restriction.

Fig.5.6 Relationship between mycorrhizal colonisation and root system dry weight, in plants harvested after one or two year's growth. Plant numbers are indicated.



Shoot dry weight appeared to be closely related to mycorrhizal colonisation, except for one outlier. The shoot dry weight showed similar responses to mycorrhizal colonisation in both the first and second season.

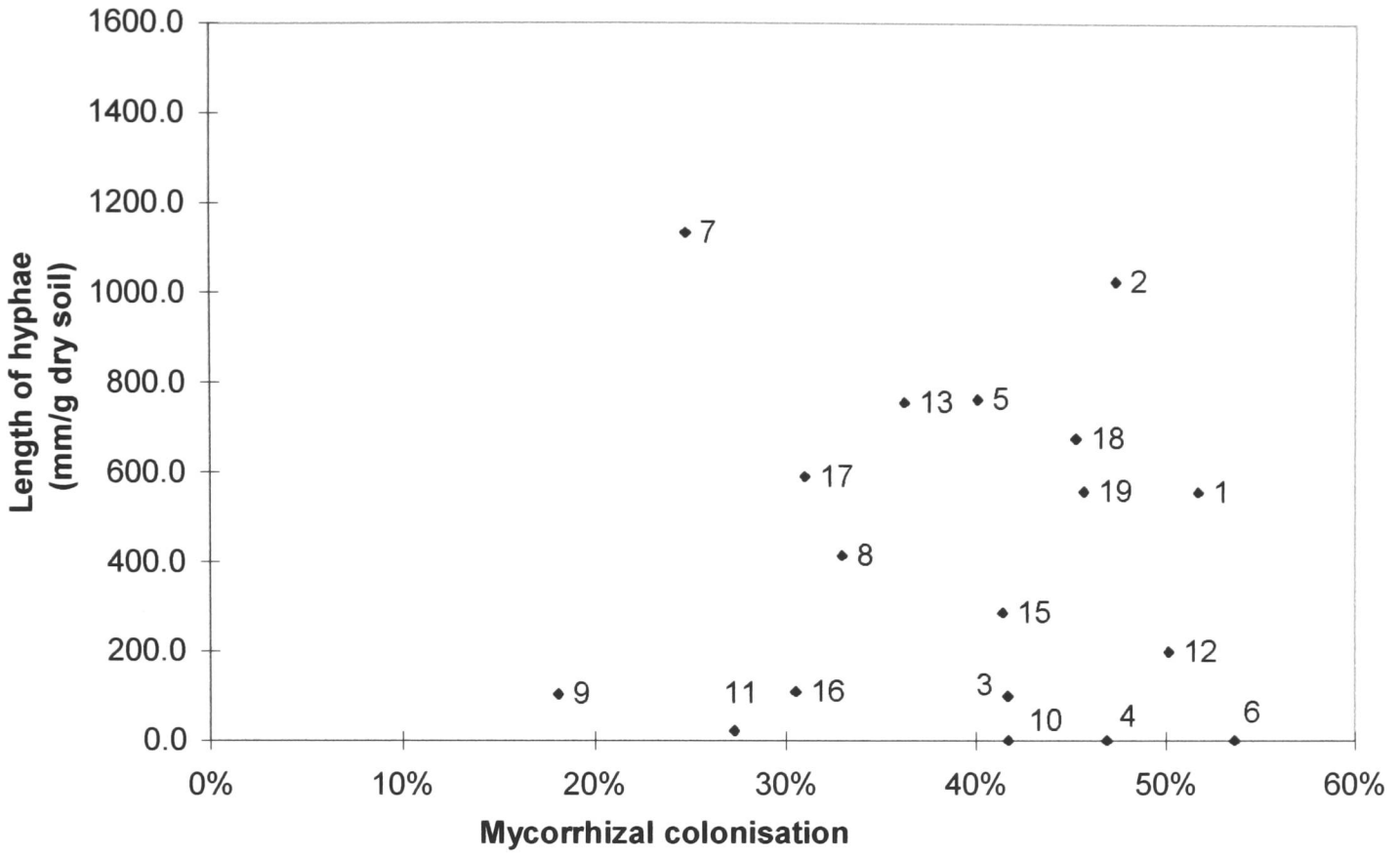
Fig.5.7 Relationship between mycorrhizal colonisation and shoot system dry weight, in plants harvested after one or two year's growth. Plant numbers are indicated.



5.3.3 Extramatrical hyphal length

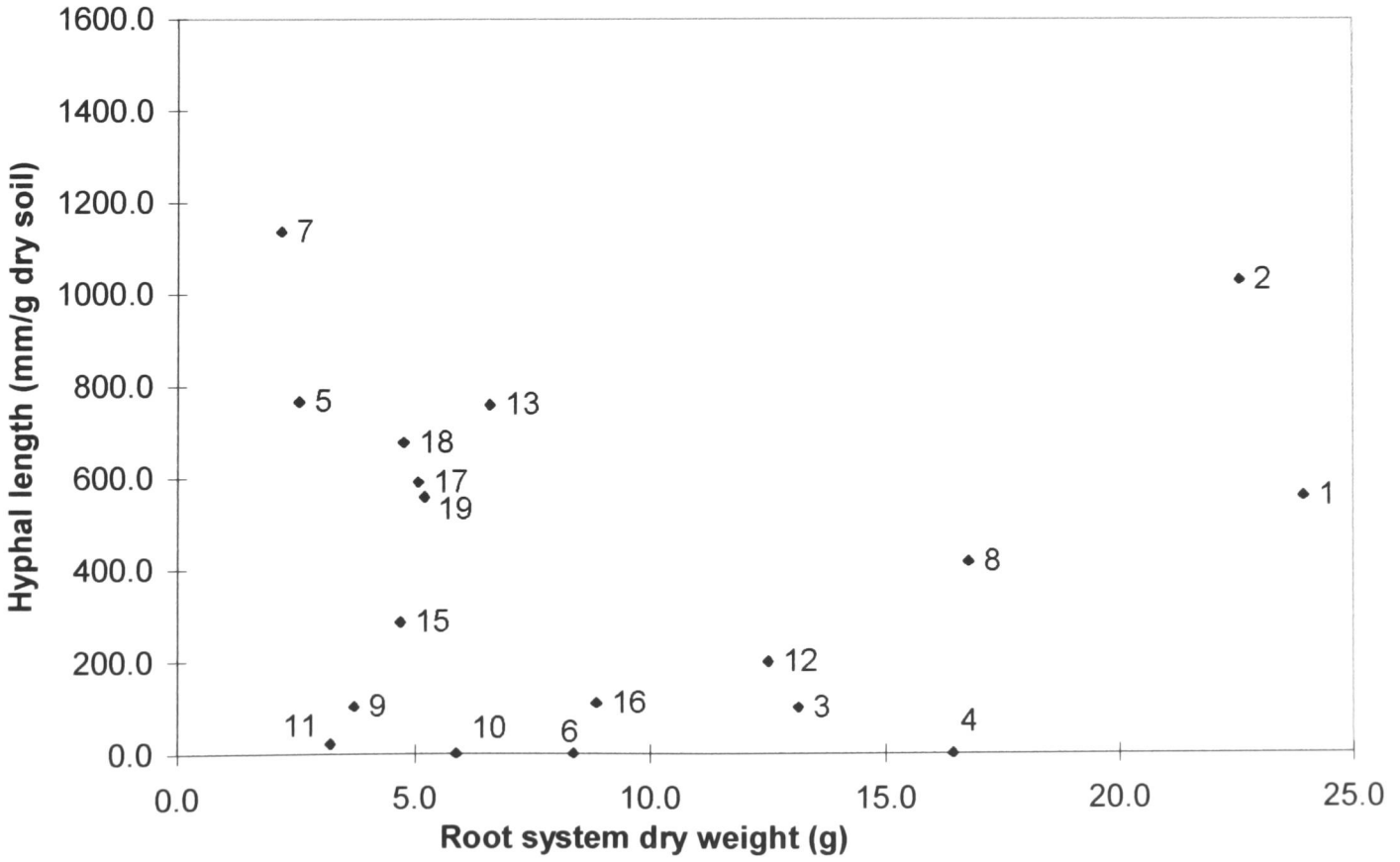
The relationship between the length of hyphae found in the hyphal section of the rhizobox and the percentage mycorrhizal colonisation of the host plant is presented in Fig.5.8. This data is shown with the length of hyphae found in an uninoculated control rhizobox, subtracted from the total values. The length of hyphae in the control rhizobox was taken to be the length of non-AM fungi, contaminating the rhizoboxes. The relationship between mycorrhizal colonisation and extramatrical hyphal length in the hyphal section of the rhizobox was poor, although there appeared to be a general trend of increasing external hyphal length with increasing mycorrhizal colonisation.

Fig.5.8 Relationship between mycorrhizal colonisation and AMF hyphal length in the hyphal section of rhizoboxes, calculated after subtracting length of other fungi. Plant numbers are indicated



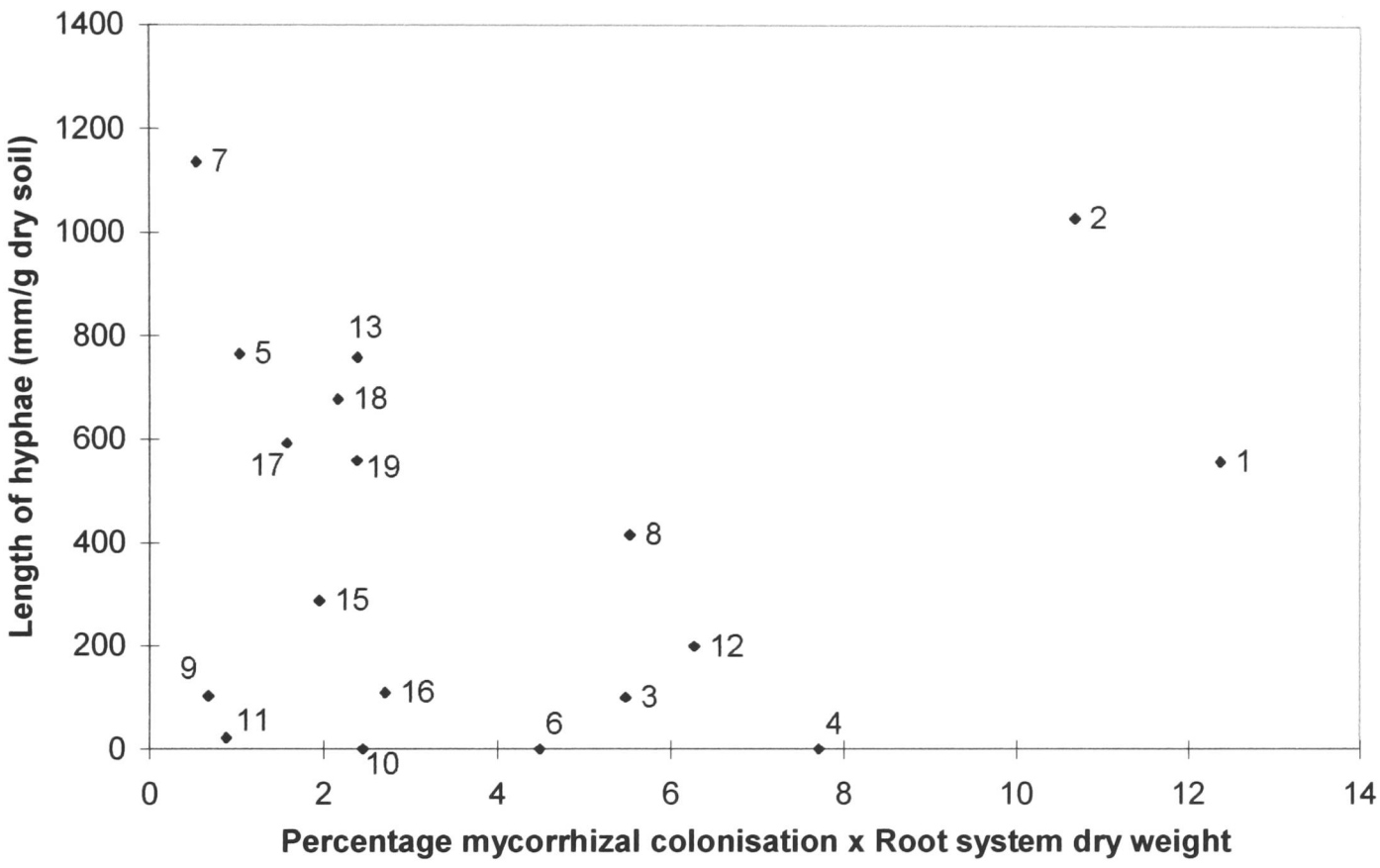
In addition there was no clear relationship between dry root weight and the length of extramatrical hyphae found (Fig.5.9).

Fig.5.9 Relationship between root system dry weight and AMF hyphal length in the hyphal section of rhizoboxes. Plant numbers are indicated



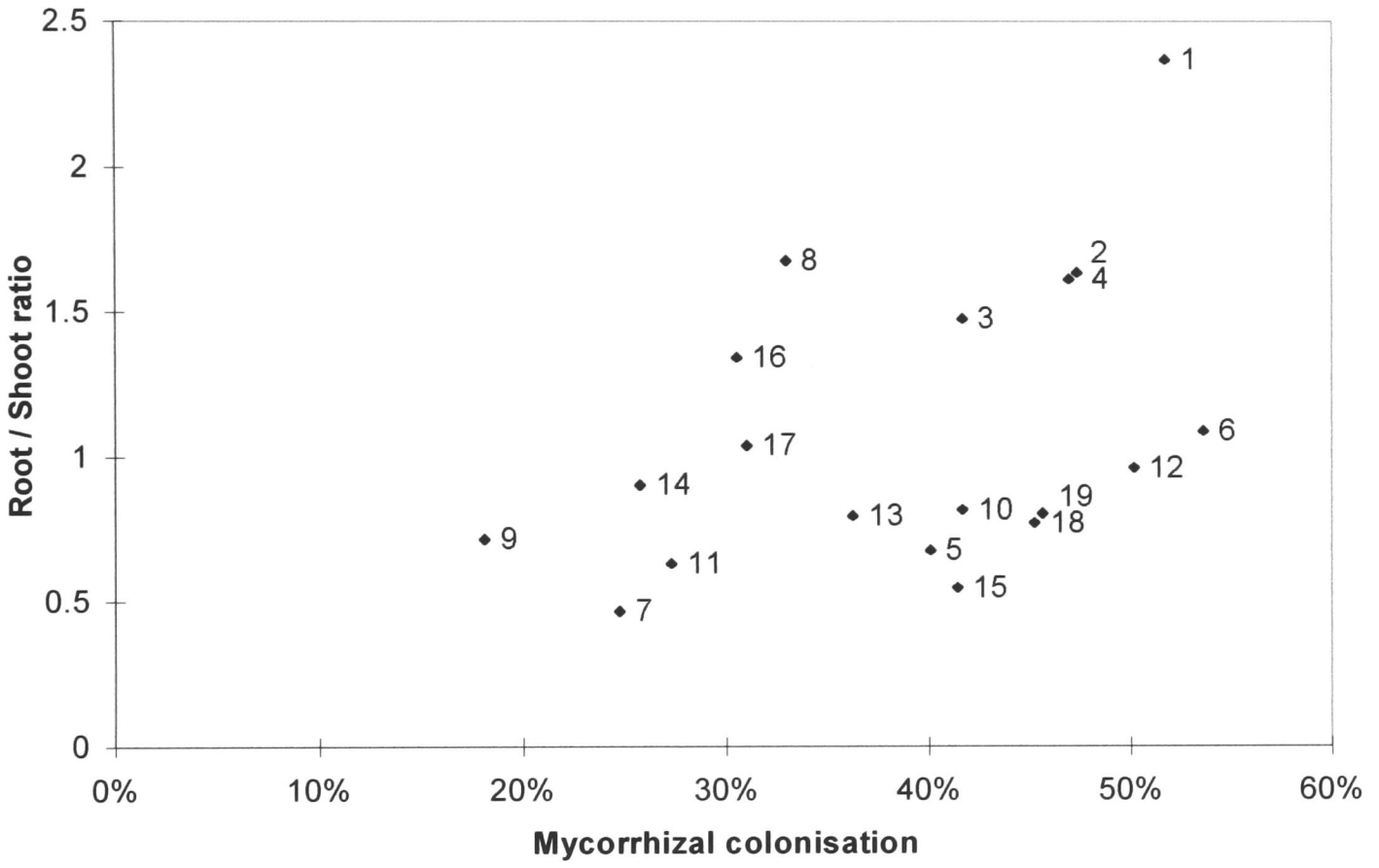
The root dry weight and mycorrhizal colonisation were multiplied as a measure of the total mycorrhizal tissue in the plant root system. This was compared to the external hyphal length (Fig.5.10). It was found that there was a negative relationship between hyphal length and total mycorrhizal colonisation of the whole root system. It appeared that external hyphal production was inversely related to the internal mycorrhizal tissue. This was not significantly correlated.

Fig.5.10 Relationship between external hyphal length and total mycorrhizal root tissue. Plant numbers are indicated.



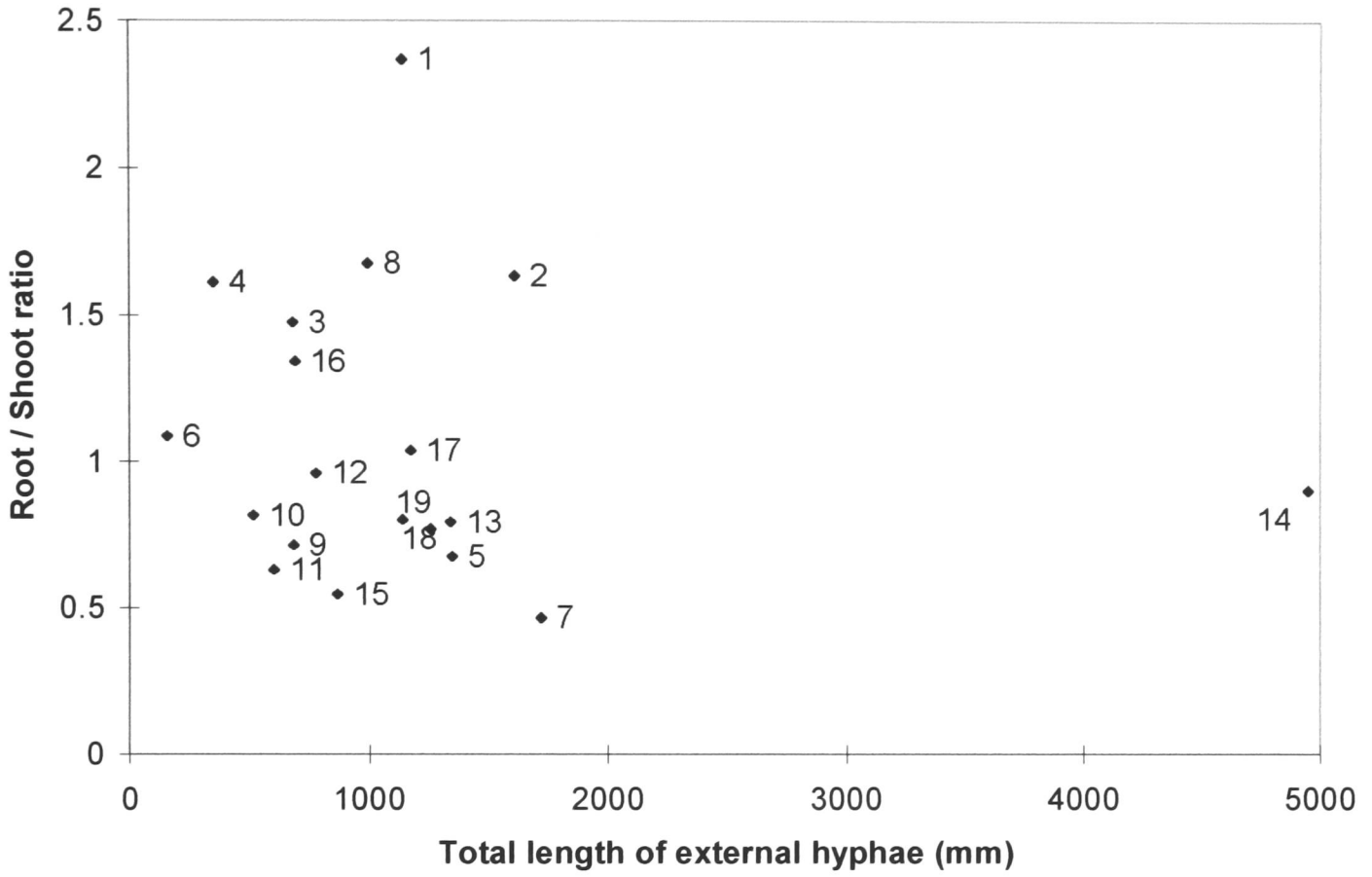
The root:shoot ration was compared with the percentage mycorrhizal colonisation of the root system (Fig.5.11). As mycorrhizal colonisation increased, the root system dry weight increased relative to the shoot dry weight. This relationship was found to be not significantly correlated.

Fig.5.11 Relationship between root:shoot ratio and mycorrhizal colonisation. Plant numbers are indicated.



The root/shoot ratio was also compared with the length of external hyphae, and a negative relationship was found. In this case as the length of external hyphae increased the dry weight of the shoot increased relative to the root system. Again this relationship was not significantly correlated (Table 5.4).

Fig.5.12 Relationship between root:shoot ratio and length of external hyphae. Plant numbers are indicated.



The correlation between these characteristics is given in Table 5.4.

Table 5.4 Correlation coefficient (r) of fungal and plant characteristics

	%Mycorrhizal colonisation	Total hyphal length	Dry weight roots	Dry weight shoot	Root/shoot ratio	Mycorrhizal colonisation x dry weight roots
%Mycorrhizal colonisation	1.00					
Total hyphal length	-0.34	1.00				
Dry weight roots	0.40	0.23	1.00			
Dry weight shoot	0.22	0.59	0.77	1.00		
Root/shoot ratio	0.43	-0.11	0.88	0.39	1.00	
Mycorrhizal colonisation x dry weight roots	0.60	0.02	0.95	0.63	0.89	1.00

n=19

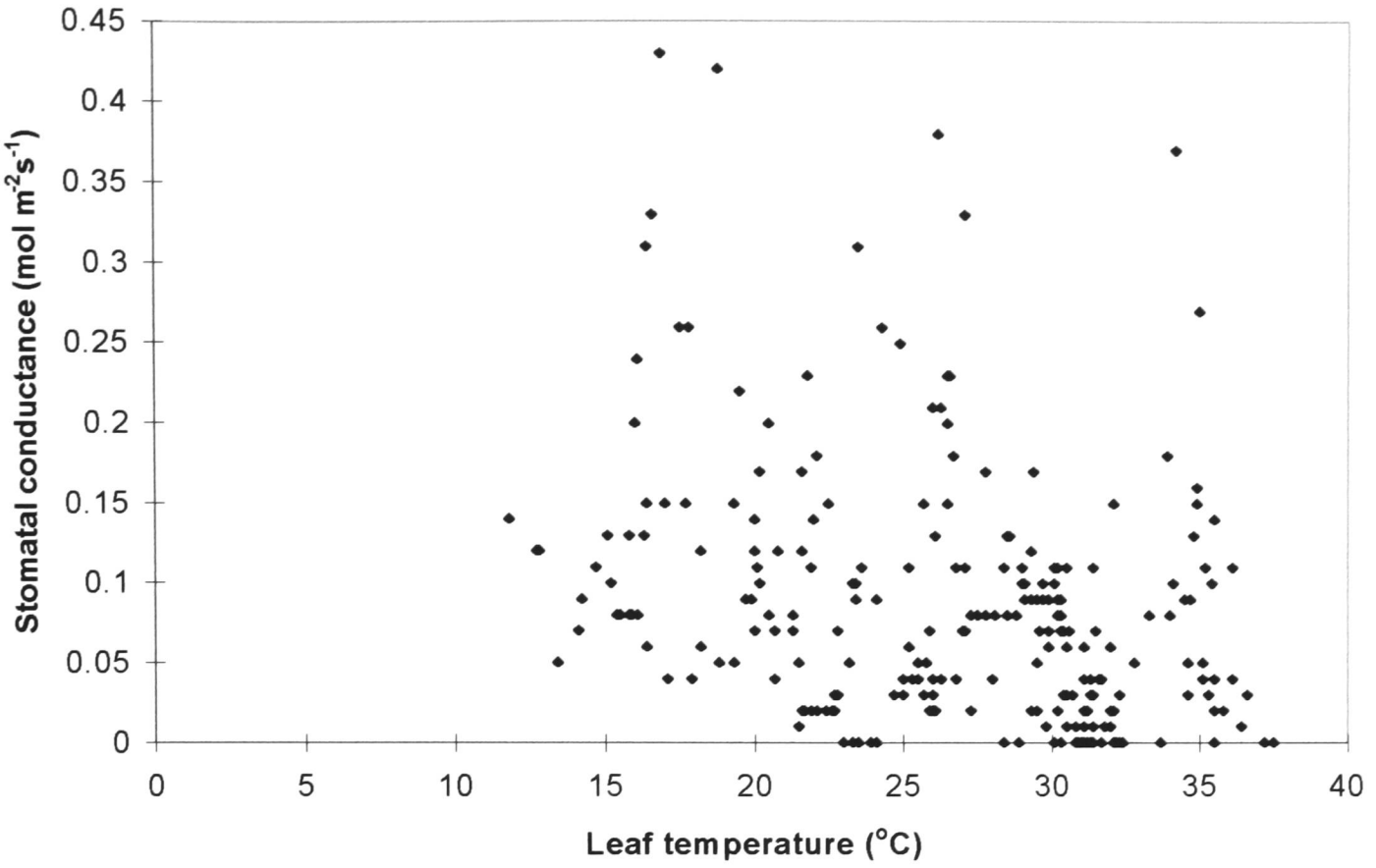
Values of r required for significance at conventional levels, on (n-2) degrees of freedom; 5%: 0.4555, 1%: 0.5751, 0.1%: 0.6932

5.3.4 Plant shoot response to growth in a rhizobox

Variations in the plants' response, in terms of the shoot processes, stomatal conductance, transpiration and photosynthesis, to water availability, access to external hyphae and vigour of external hyphae are examined in sections 5.3.5 to 5.3.7. The replicate plants are shown in sections 5.3.1 and 5.3.2 to vary greatly in size and mycorrhizal colonisation. For this reason the overall response of the shoots to changes in the aerial environment were examined for typical behaviour. Plant shoot responses in relation to water availability are discussed in general in Chapter 1. The shoot responses to environmental conditions in mycorrhizal plants are examined below in order to assess whether they can be considered as a norm, in comparison to non-mycorrhizal plants. The relationship between the various shoot processes both with each other and with temperature are given in Figs.5.13-5.17. The correlation between processes is summarised in Table 5.5.

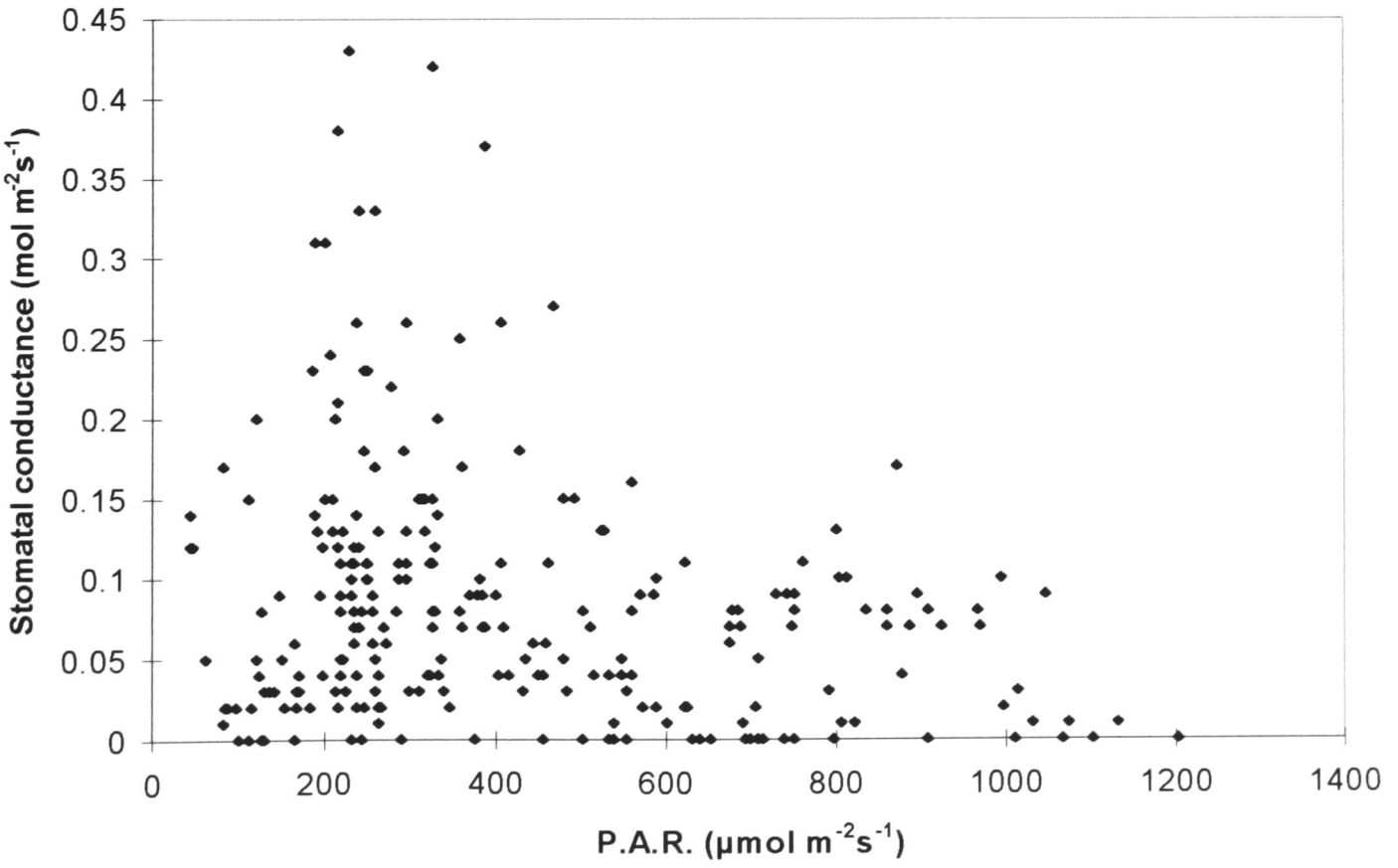
Table 5.5 shows a small decrease in leaf stomatal conductance with increasing air temperature, but the distribution of points in the scatterplot of stomatal conductance versus temperature is largely random in Figure 5.13. There was no clear effect of temperature on stomatal conductance. The temperature in the glasshouse was maintained between 12 to 37°C. Between 12 to 22°C stomatal conductance was never zero, but at higher temperatures, stomatal conductance was generally decreased and often zero.

Fig.5.13 Relationship between stomatal conductance and air temperature



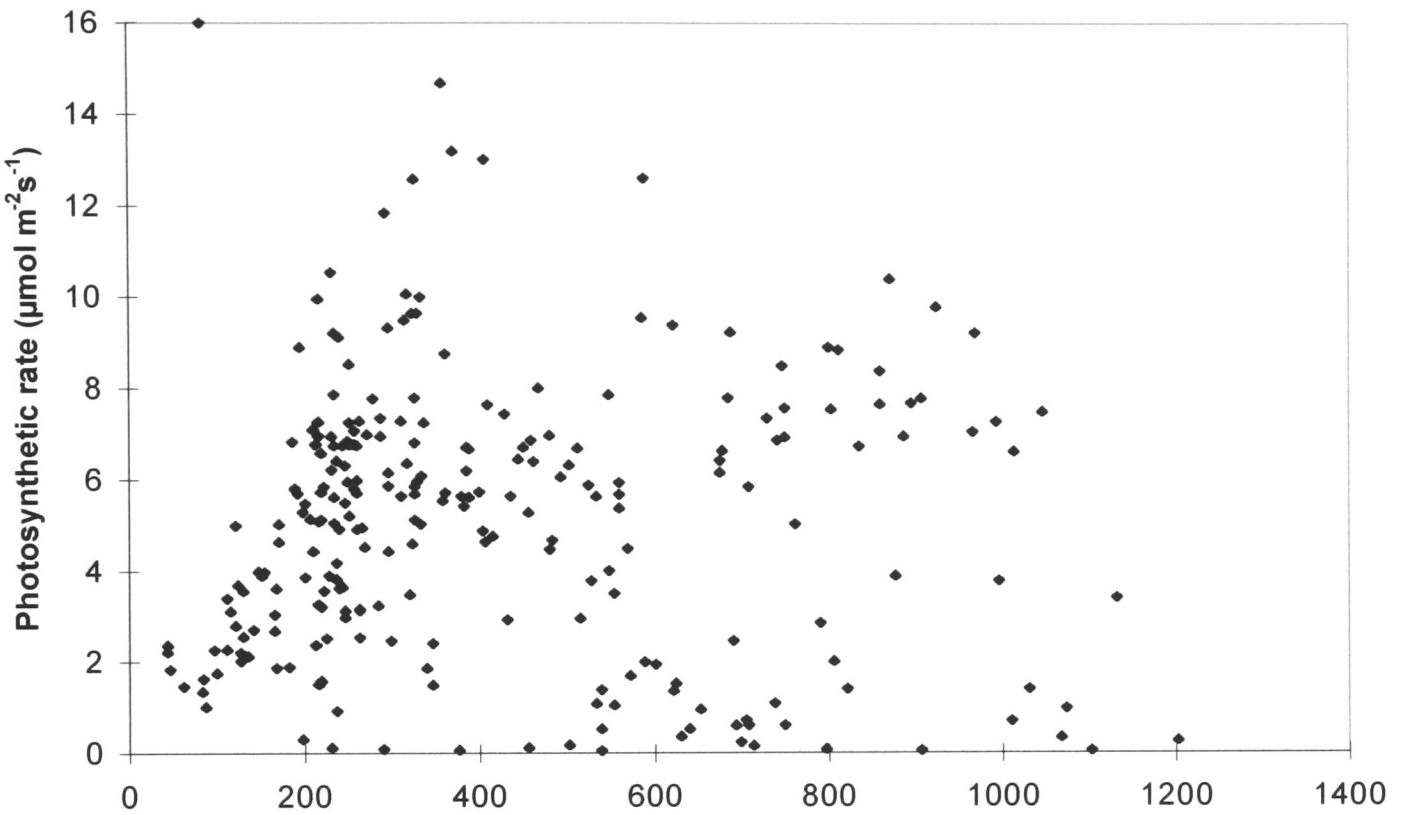
There was a relatively small change in stomatal conductance of 0-0.2 mol $m^{-2}s^{-1}$ over a broad range of photosynthetically active radiation (P.A.R.) from 100-1000 µmol $m^{-2}s^{-1}$ (Fig.5.14). There was maximum stomatal conductance between 200 and 500 mol m^{-2} s^{-1} P.A.R.

Fig.5.14 Relationship between stomatal conductance and photosynthetically active radiation



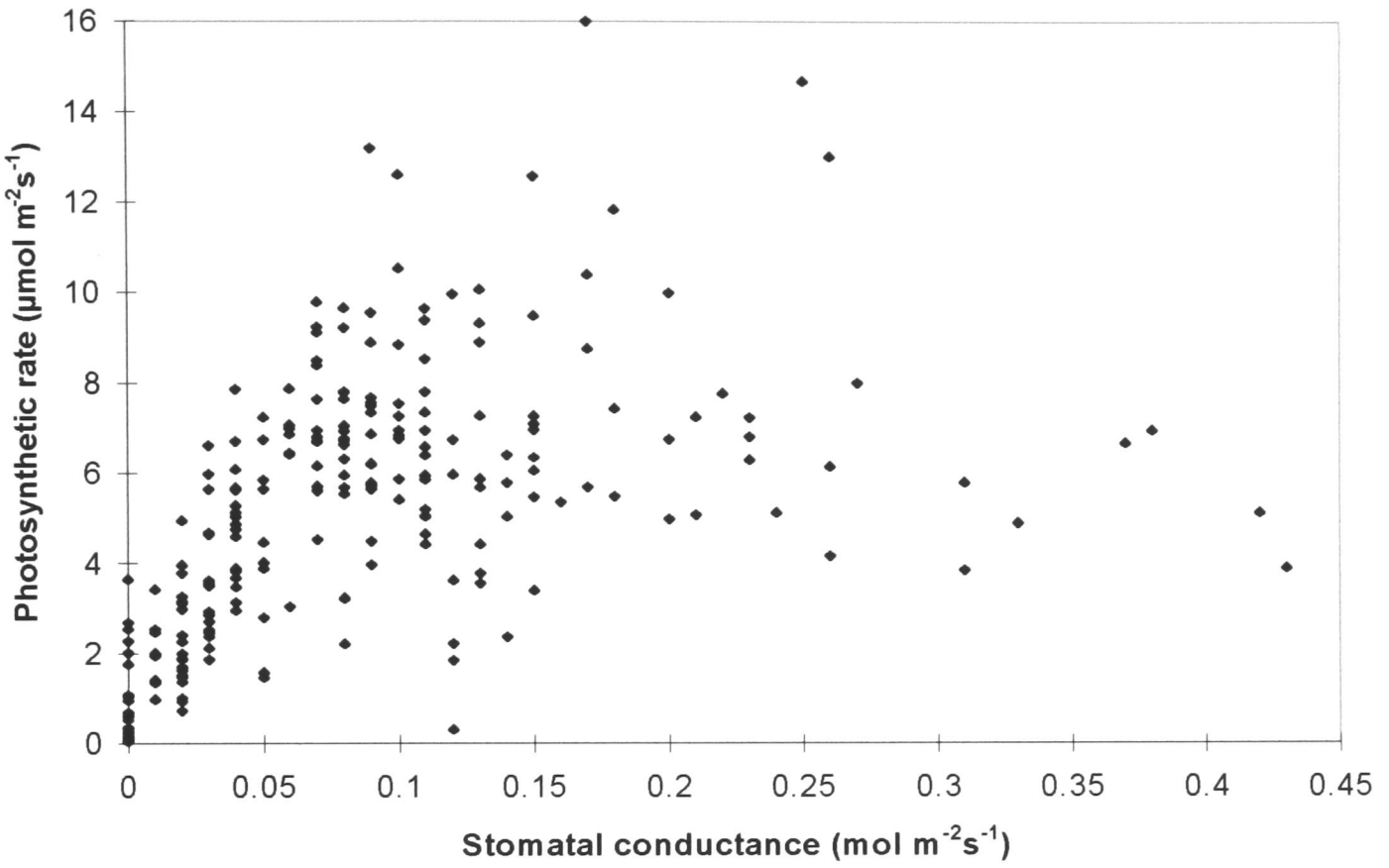
Photosynthesis was positively related to photosynthetically active radiation (Fig.5.15). Photosynthetic rate appeared to be directly correlated to photosynthetically active radiation below 200 μmol $m^{-2}s^{-1}$ and then reached an upper limit of approximately 15 μmol $m^{-2}s^{-1}$.

Fig.5.15 Relationship between photosynthetically active radiation (P.A.R.) and photosynthetic rate



Stomatal conductance and photosynthesis were positively correlated (Table 5.5). The relationship between these two parameters appeared to follow a hyperbolic curve. There was a steady increase in photosynthetic rate until a stomatal conductance of 0.1 mol $m^{-2}s^{-1}$, reaching a maximum of approximately 10 µmol $m^{-2}s^{-1}$ (Fig.5.16).

Fig.5.16 Relationship between stomatal conductance and photosynthesis



This positive correlation was also seen between stomatal conductance and transpiration (Fig.5.17). The variation in transpiration with stomatal conductance increased as stomatal conductance increased. Transpiration was more closely related to stomatal conductance when the stomatal conductance was low. The relationship between transpiration and stomatal conductance was the most closely correlated of all parameters at r =0.8 (Table 5.5).

Fig.5.17 Relationship between transpiration and stomatal conductance

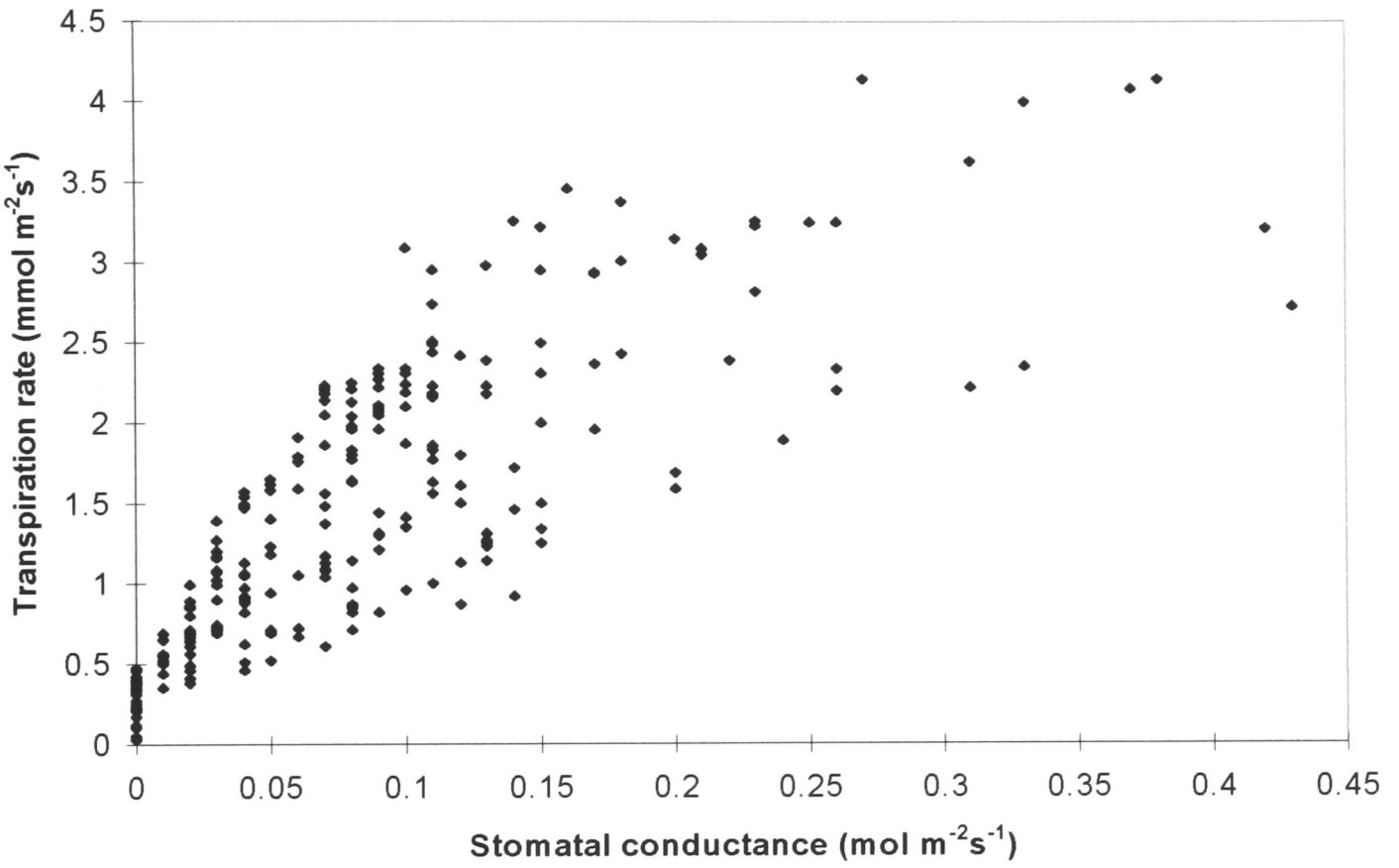


Table 5.5 Correlation coefficients (r) of leaf parameters and atmospheric conditions.

	P.A.R. ($\mu molm^{-2}s^{-1}$)	Leaf temperature (^{o}C)	Transpiration rate ($mmol\ m^{-2}s^{-1}$)	Stomatal conductance ($mol\ m^{-2}s^{-1}$)	Photosynthetic rate ($\mu molm^{-2}s^{-1}$)
P.A.R.	1.00				
Leaf temperature	0.59	1.00			
Transpiration rate	0.01	0.13	1.00		
Stomatal conductance	-0.25	-0.32	0.80	1.00	
Photosynthetic rate	-0.03	-0.17	0.65	0.50	1.00

n=249

Values of r on (n-2) degrees of freedom required for significance at conventional levels;

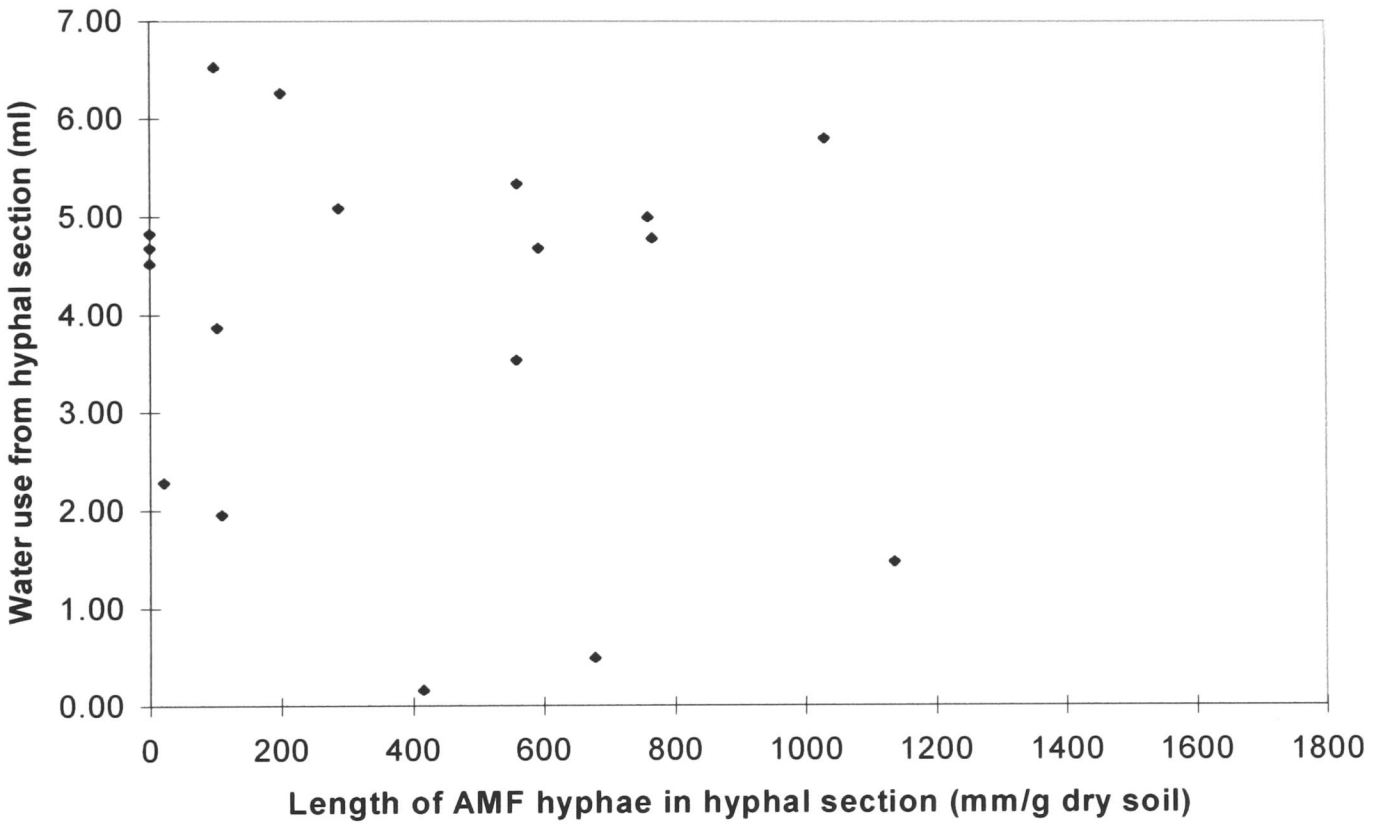
5%: 0.1946, 1%: 0.2540, 0.1%: 0.3211

The correlation coefficient indicates the likelihood of a linear relationship between the measured variables. However these relationships are not always linear, as indicated in the scatter graphs, and the values for the correlation coefficient should be considered in combination with the graphs. The temperature of the leaves and the photosynthetically active radiation were closely related and positively correlated, significant at the 0.1% level. Stomatal conductance was negatively correlated to both photosynthetically active radiation and leaf temperature at 1% significance. Photosynthesis was also positively correlated with stomatal conductance (0.1%) but to a much lesser extent than transpiration and stomatal conductance (0.1%). Photosynthesis was only poorly related to either photosynthetically active radiation (NS) or leaf temperature (NS). Photosynthesis and transpiration were positively correlated (0.1%).

5.3.5 Experiment (A): Relationship of water uptake from soil with extramatrical hyphae

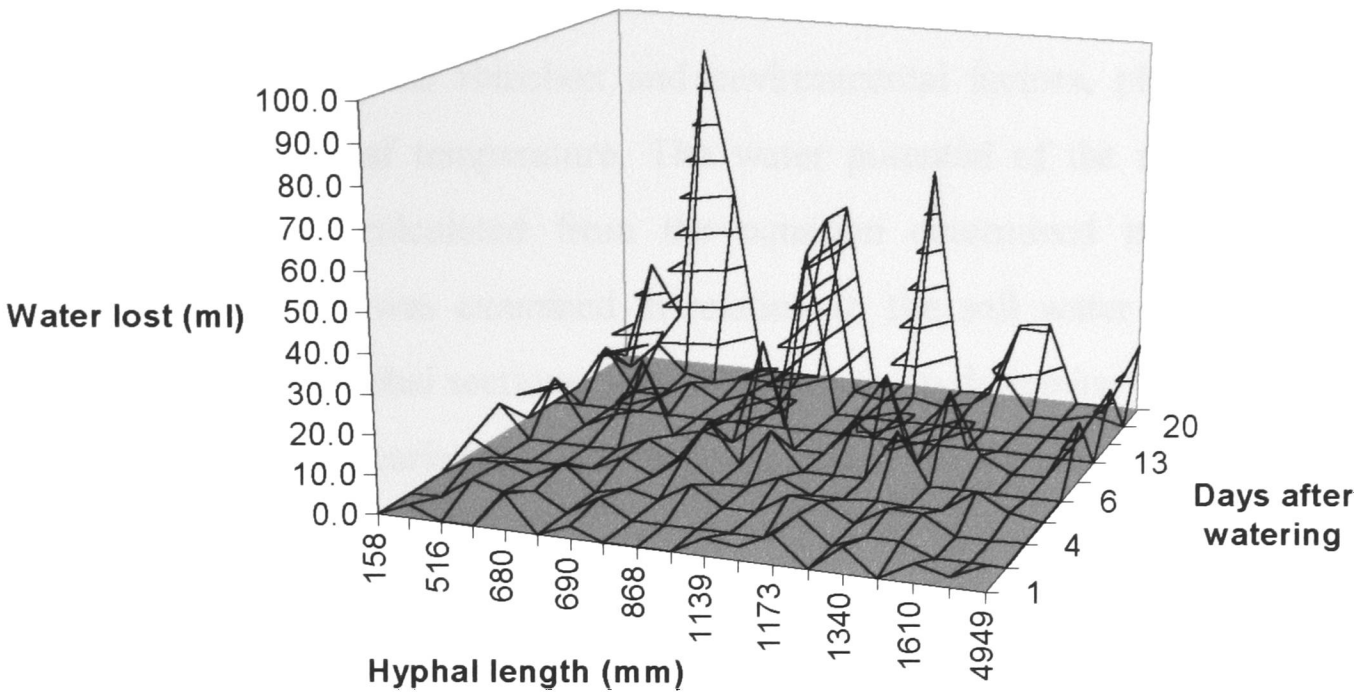
The uptake of water from the section of the rhizoboxes containing solely hyphae was compared with the quantity of hyphae in that section, to assess whether direct transport of water could be observed. No clear relationship was found between the length of hyphae extracted from the hyphal section and the volume of water lost from the hyphal section over a five day period (Fig.5.18). There was however a trend of higher water loss with increased hyphal length.

Fig.5.18 Relationship between AMF hyphal length in the hyphal section of rhizoboxes and water loss from hyphal sections in five days



When this relationship was examined over a longer period of time, there appeared to be no relationship between the external hyphal length and removal of water from the hyphal section (Fig.5.19). There was greater water removal the longer water was withheld from the hyphal section, but only in some rhizoboxes.

Fig.5.15 Relationship of water removal from hyphal section of rhizoboxes with time and hyphal length.



Shoot response to differences in the soil water content of each rhizobox section

The frequency of plants which showed a higher or lower percentage difference in transpiration when there was a difference in water content of the rhizobox sections was recorded (Table 5.6). Percentage difference in transpiration was compared with wetter and drier root+hyphal section water content relative to hyphal section, and analysed using a chi^2 test. There was no significant relationship, indicating that relative transpiration and relative difference in water content were independent.

Table 5.6 Frequency of plants with transpiration greater or less than control, compared to soil water content of root+hyphal and hyphal section of rhizobox, where control is equal soil moisture content in each rhizobox section.

Group	Transpiration >control	Transpiration <control
Roots+hyphae wet	10	3
Hyphae wet	28	29

chi^2 test
p=0.51

Shoot response to soil water availability in rhizobox sections and aerial environment

The data from all replicate plants in the first season was used to compare the changes in leaf processes relative to the soil water potential of the root+hyphal and the hyphal sections of the rhizobox and environmental factors, photosynthetically active radiation and leaf temperature. The water potential of the root+hyphal and hyphal sections was calculated from the equation determined in section 5.2.1. Stomatal conductance was examined in relation to the soil water potential in the root+hyphal and the hyphal sections of the rhizoboxes, to determine which were most useful in explaining the variation shoot response. These are summarised in Table 5.7.

Table 5.7 Relationship between stomatal conductance and P.A.R., leaf temperature and soil water potential in the root+hyphal and hyphal sections of the rhizobox, in the first season of growth.

	Coefficients	P-value
Intercept	0.13	0.03
PAR (μmol $m^{-2}s^{-1}$)	-0.0001	0.08
Leaf temp (^{O}C)	-0.0007	0.7
φ (root+hyphae) (MPa)	0.043	0.01
φ (hyphae) (MPa)	-0.031	0.9
Adjusted R Squared	0.12	

Stomatal conductance was positively related to soil water potential in the root+hyphal section. It was negatively correlated with soil water potential in the hyphal section. Stomatal conductance was also negatively related to leaf temperature and P.A.R. These relationships were only significant at the 5% level for soil water potential in the root+hyphal section of the rhizobox. Stomatal conductance responded to changes in the soil water availability in the root+hyphal section but did not appear to be influenced by soil water availability in the hyphal section, nor by measured atmospheric conditions.

5.3.6 Experiment (B): Removal of hyphal section.

Hyphae in the hyphal section of the rhizobox, were severed from the root system in the root+hyphal section. Changes in the leaf processes were recorded continuously over a 20 minute period. After the first 10 minutes the root+hyphal section and the hyphal section were severed. The monitoring was resumed for a further 10 minutes, 90 minutes after severing. There were four replicate plants at this point. The mean changes in shoot processes for all replicate plants with time are shown in Fig.5.20. The leaf processes of each plant are then shown separately because of their varied responses (Figs.5.21-5.23).

There was greatest change in the photosynthetic rate of the plants. It declined before and 90 minutes after severing but remained constant immediately afterwards. There was a smaller change in transpiration, which also remained stable immediately after severing and then continued to decline 90 minutes later. There was very little change in the stomatal conductance of the plants. It was initially stable and then rose again 90 minutes later. Photosynthesis showed little relationship with stomatal conductance in all replicates.

Fig.5.20 Mean gas exchange before, immediately after and 90 minutes after severing external hyphae from roots

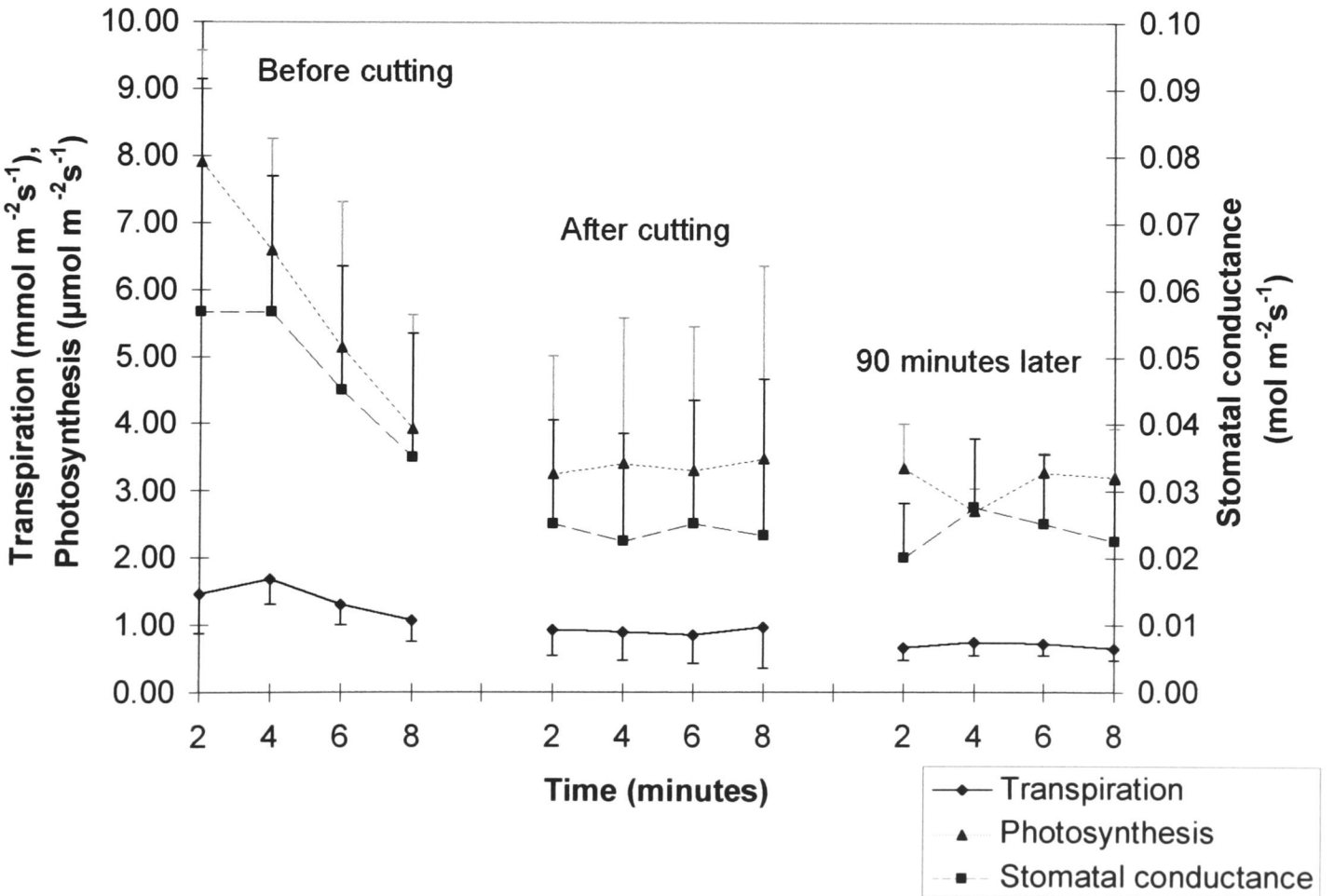
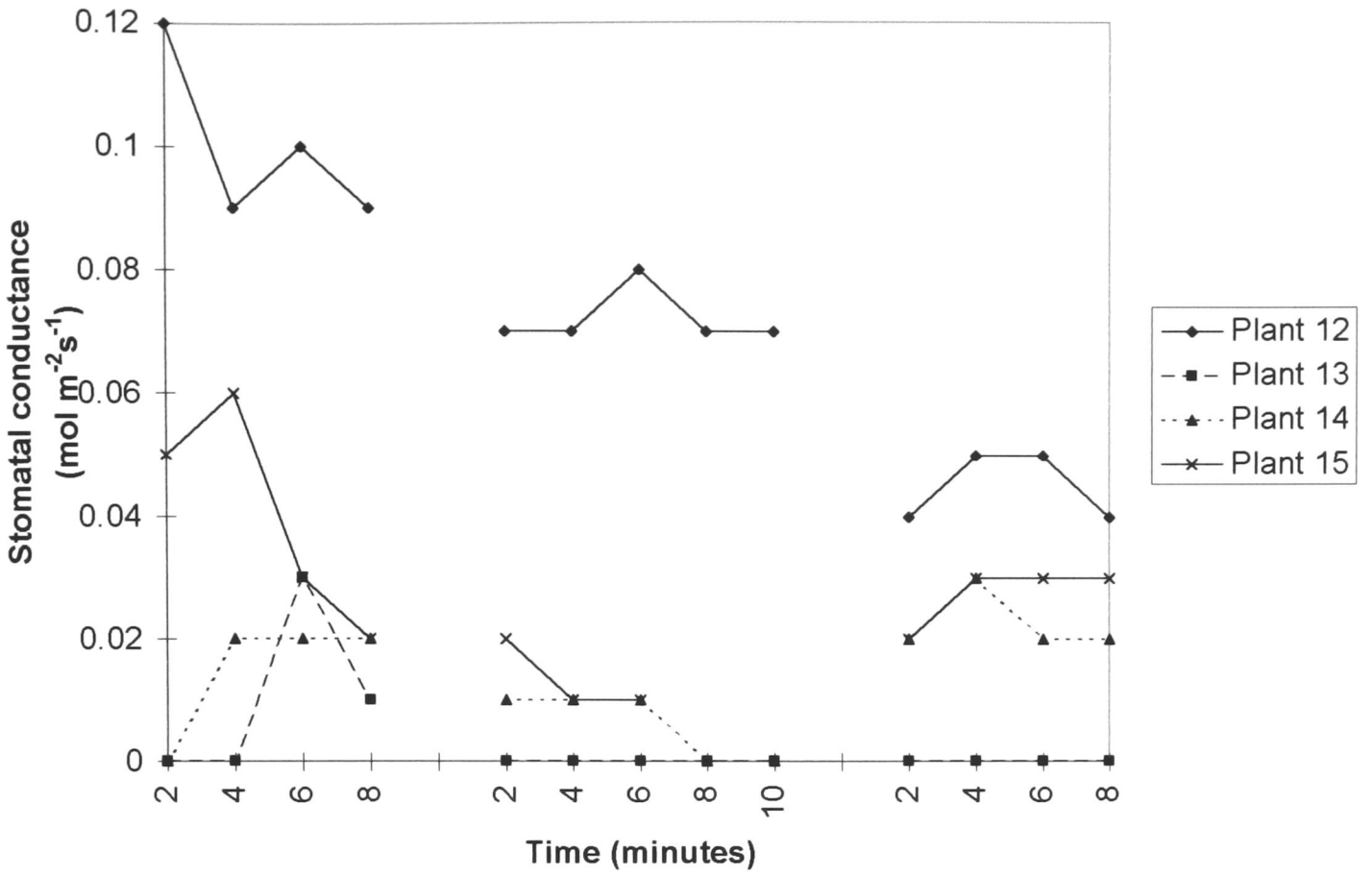


Fig.5.21 Changes in stomatal conductance showing differing replicate responses



Stomatal conductance showed a decrease in plant 12, and increase in plants 14 and 15 and no change in plant 13.

Fig.5.22 Changes in transpiration showing differing replicate responses

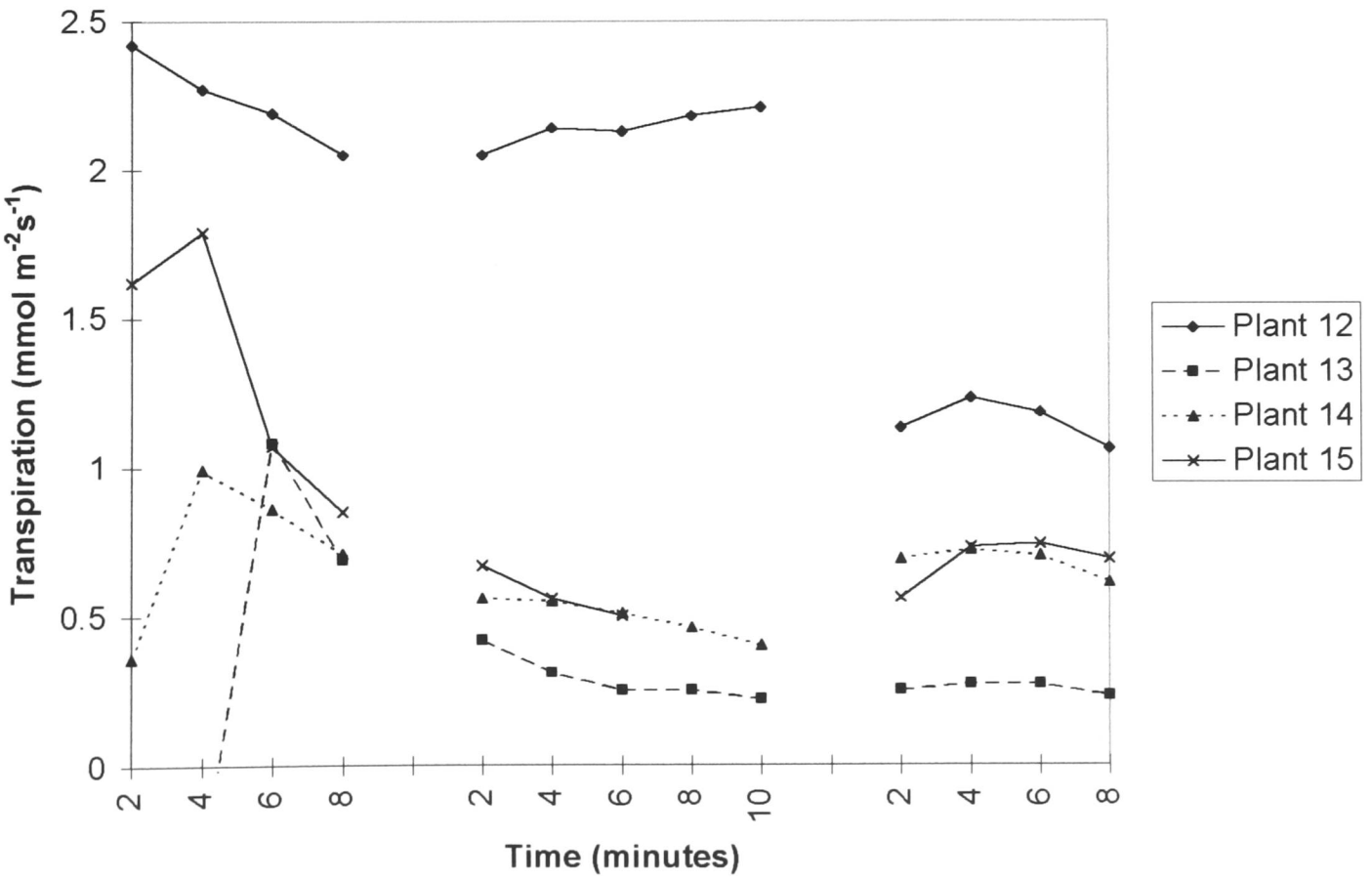
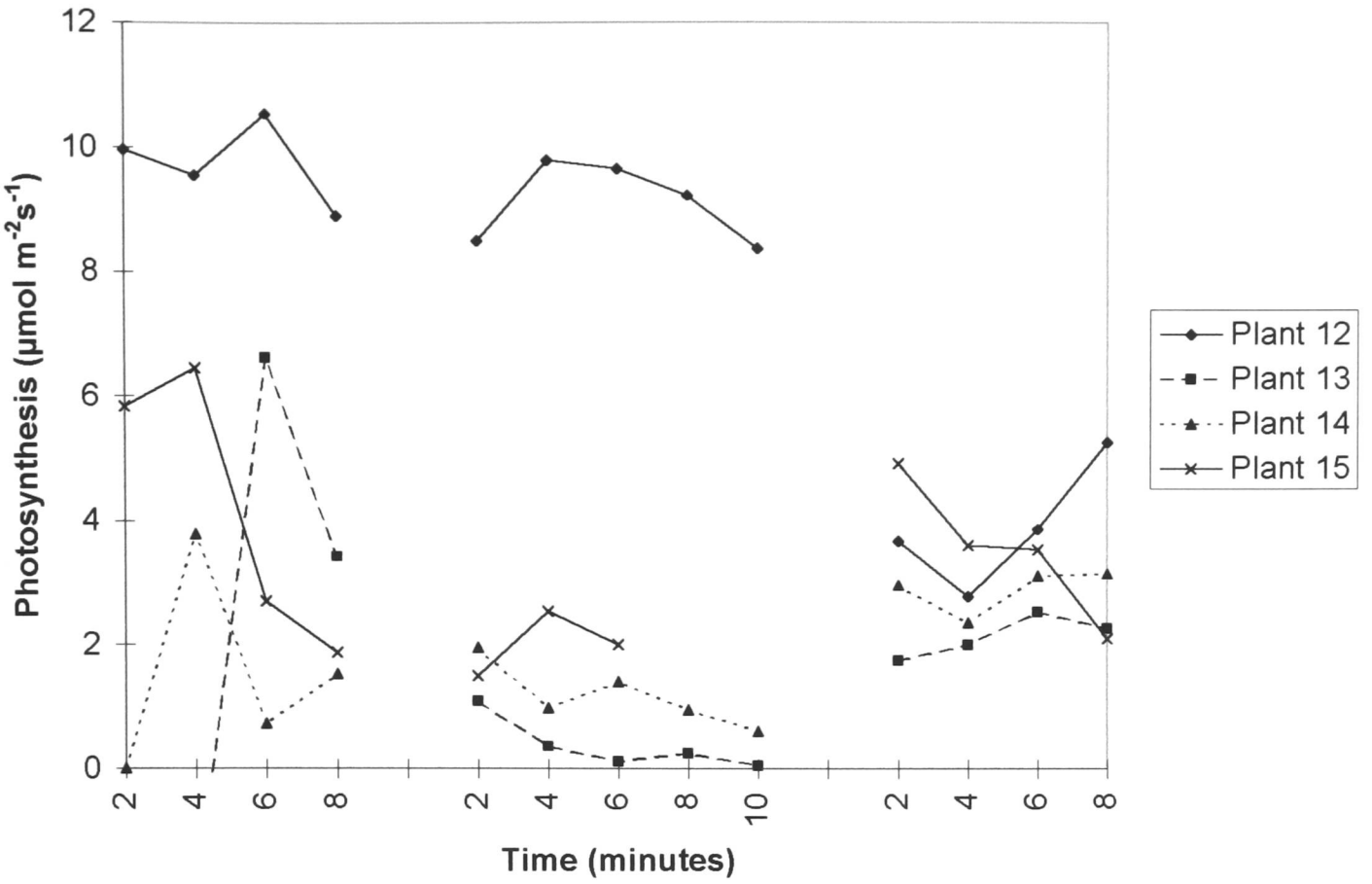


Fig.5.23 Changes in photosynthesis showing differing replicate responses

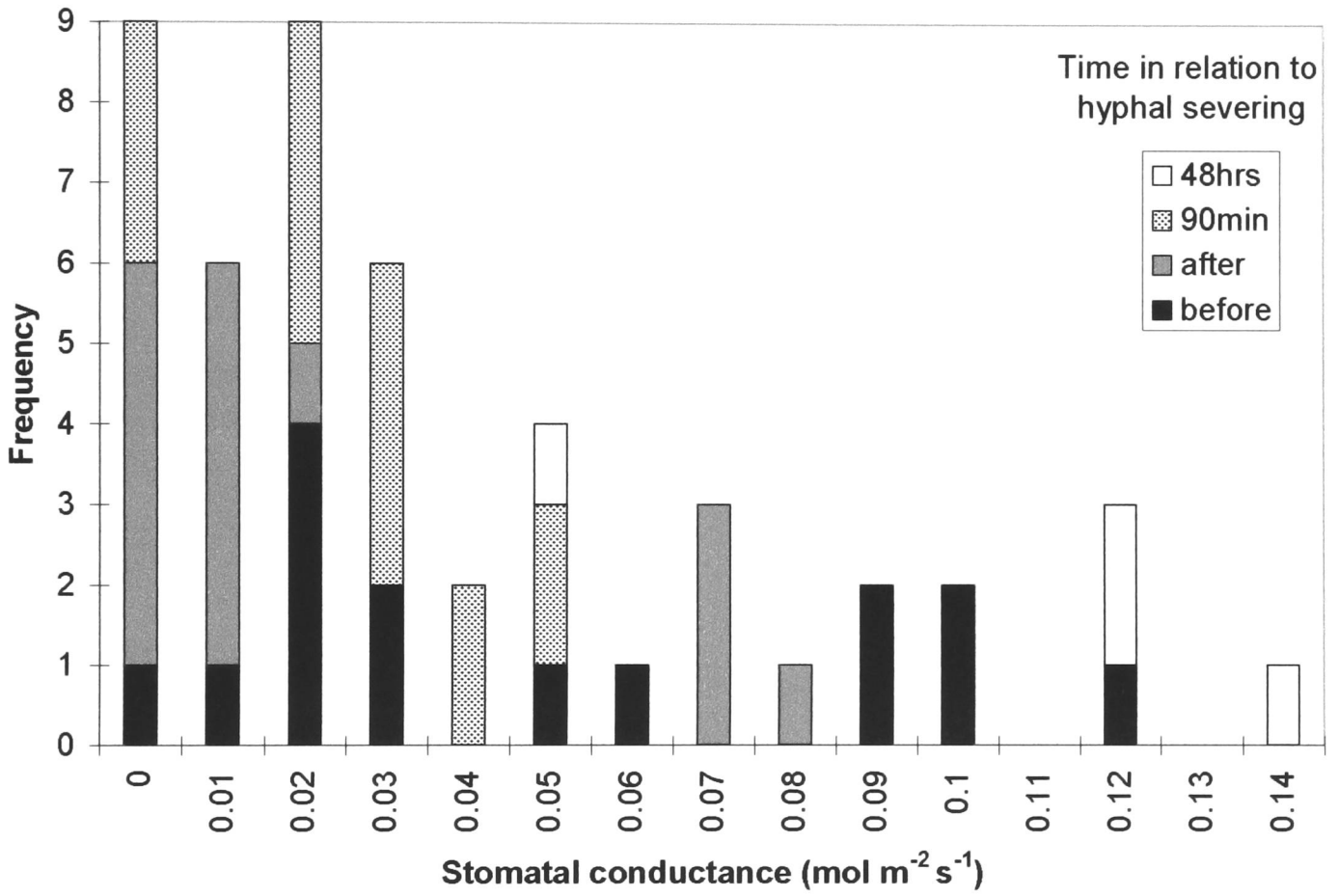


In the 10 minutes after cutting the hyphae, transpiration continued to decline in plant 13-15 but increased in plant 12 (Fig.5.22). Photosynthesis continued to decrease in plants 13 and 14, increased in plant 15, and remained relatively stable in 12 (Fig.5.23). The declines in transpiration and photosynthesis did not occur in the same plants.

The leaf chamber was removed for 90 minutes and then leaf processes were measured again. Plants 12, 14 and 15 showed an increase in transpiration, although not to a value as great as the rate at the beginning of the experiment. In plant 13, transpiration was stable. In plant 12, photosynthesis was also reduced. In the rest of the plants, photosynthesis was increased.

The leaf processes were measured again after 48 hours. The changes in stomatal conductance are shown in Fig.5.24. Before severing there is a broad range of stomatal conductances from 0 to 0.12 mol $m^{-2}s^{-1}$. However after severing the range is reduced to lower values with a maximum of 0.08 mol $m^{-2}s^{-1}$ and 0.05 $m^{-2}s^{-1}$. Only 48 hours after severing have the stomatal conductances increased to their original values and the range is at higher values than before severing. This change was found to be significant at the 5% level using a Kolmogorov-Smirnov test.

Fig.5.24 Frequency of stomatal conductance before and after hyphal severing



Change in shoot response related to external hyphal length

The relationship between total external hyphal length in the hyphal section and plant shoot response is summarised in Table5.8. In those plants with extensive hyphal development in the hyphal section of the rhizobox, photosynthesis and transpiration were increased. In those plants with little hyphal development, photosynthesis was decreased in one plant, and increased in two. Transpiration was increased in one plant and decreased in two. The same pattern of response was also seen in stomatal conductance, where the plant with extensive hyphal development always showed an increase in conductance after severing of the hyphae.

When the length of live external hyphae were compared to the leaf processes, a pattern was seen in the change in transpiration and stomatal conductance. Plants with few hyphae showed an increase in these processes after severing, while those with extensive hyphae showed a decrease. A similar pattern as in total hyphal length was seen in photosynthesis.

Table 5.8 Number of plants with increased or decreased leaf processes after severing of hyphae, compared to relative hyphal length

Total hyphal

length	long	short		long	short		long	short
Transpiration increased	1	1	Stomatal conductance increased	1	1	Photosynthesis increased	1	2
Transpiration decreased		2	Stomatal conductance decreased		2	Photosynthesis decreased		1

Live hyphal

length	long	short		long	short		long	short
Transpiration increased		2	Stomatal conductance increased		2	Photosynthesis increased	1	2
Transpiration decreased	2		Stomatal conductance decreased	2		Photosynthesis decreased	1	

Long = above average length of hyphae
Short = below average length of hyphae
Mean total hyphal length 1984mm/g dry soil
Mean live hyphal length 144mm/ dry soil

Stomatal conductance compared to soil and aerial factors

The stomatal conductance was further compared to both the soil water potential in each section of the rhizoboxes, and the photosynthetically active radiation and leaf temperature. The data from all plants in the first season is presented again as a control for comparison with the plants subjected to the severing treatment.

Table 5.9 Linear regression of stomatal conductance against photosynthetically active radiation, leaf temperature and soil water potential in the root+hyphal and hyphal section of the rhizoboxes.

a) first season

	Coefficients	P-value
Intercept	0.1303	0.03
PAR (μmol $m^{-2}s^{-1}$)	-0.0001	0.08
Leaf temp (^{O}C)	-0.0007	0.73
φ (root+hyphae) (MPa)	0.0427	0.01
φ (hyphae) (MPa)	-0.0311	0.90
Adjusted R Square	0.12	

b) Hyphal severing treated plants

Coefficients	Before	After	90min after
Intercept	0.97	1.00	0.11
PAR (μmol $m^{-2}s^{-1}$)	0.00	0.00	0.00
Leaf temp (^{O}C)	-0.03	-0.03	0.00
φ (root+hyphae) (MPa)	0.47	0.03	-0.21
φ (hyphae) (MPa)	0.63	0.85	1.11
P-value	0.00	0.00	0.28
PAR (μmol $m^{-2}s^{-1}$)	0.03	0.95	0.00
Leaf temp (^{O}C)	0.00	0.00	0.61
φ (root+hyphae) (MPa)	0.01	0.80	0.21
φ (hyphae) (MPa)	0.01	0.00	0.00
Adjusted R Square	0.95	0.93	0.88

Over the short time period of 100 minutes for this experiment there was a strong relationship between stomatal conductance and the soil and aerial parameters. It was positively related to soil water potential in both sections. There was a negative relationship with leaf temperature, but P.A.R. appeared to have no effect. These relationships are highly significant before severing but appear less so after. The relationships between stomatal conductance and soil water potential are presented graphically in Figs.5.25 and 5.26. They show a decrease in stomatal conductance immediately after severing but no change in the relationship with soil water potential. 90 minutes later the relationship is less evident.

Fig.5.25 Relationship between stomatal conductance and soil water potential in the root+hyphal section of the rhizobox.

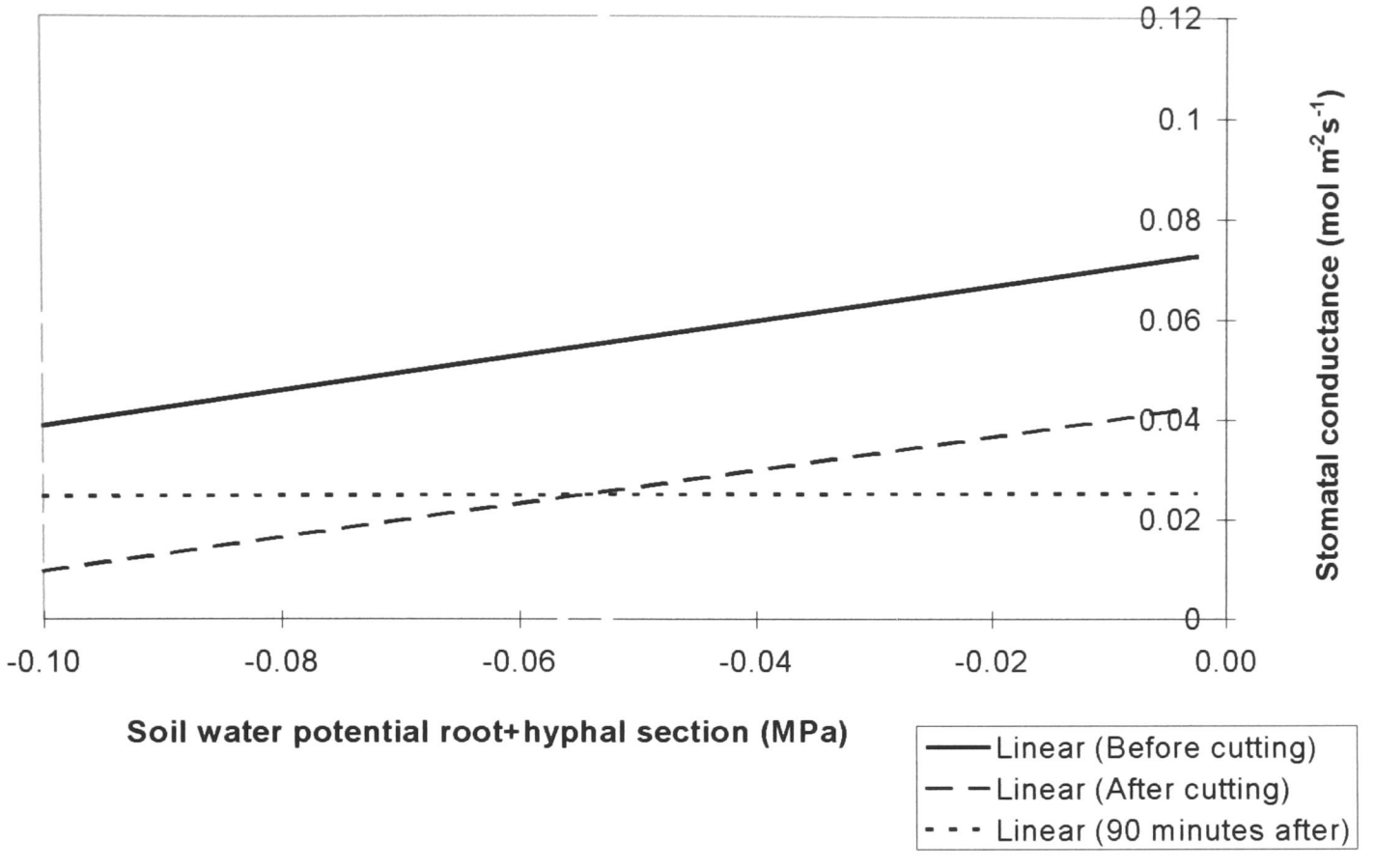
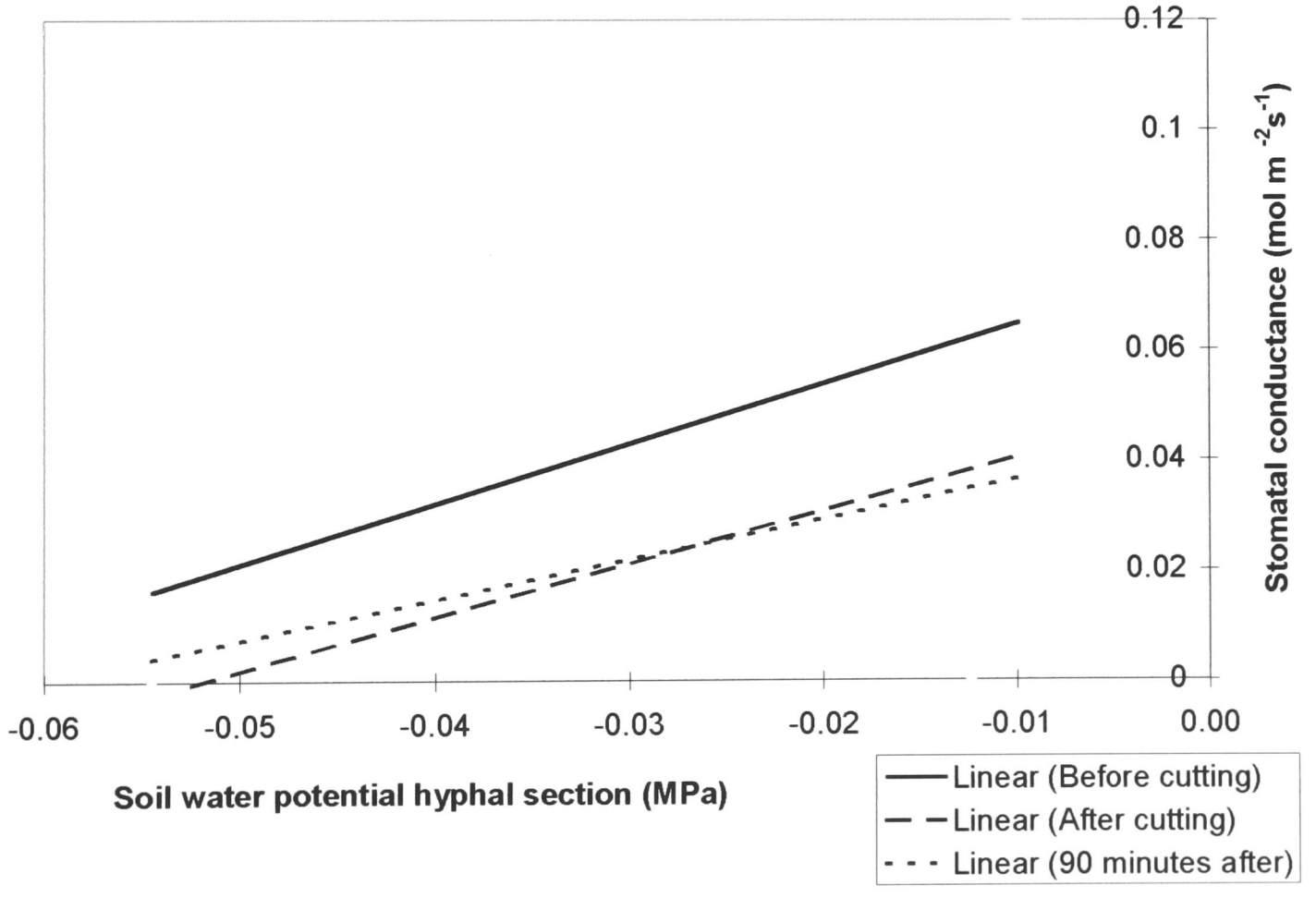


Fig.5.26 Relationship between stomatal conductance and soil water potential in the hyphal section of the rhizobox.



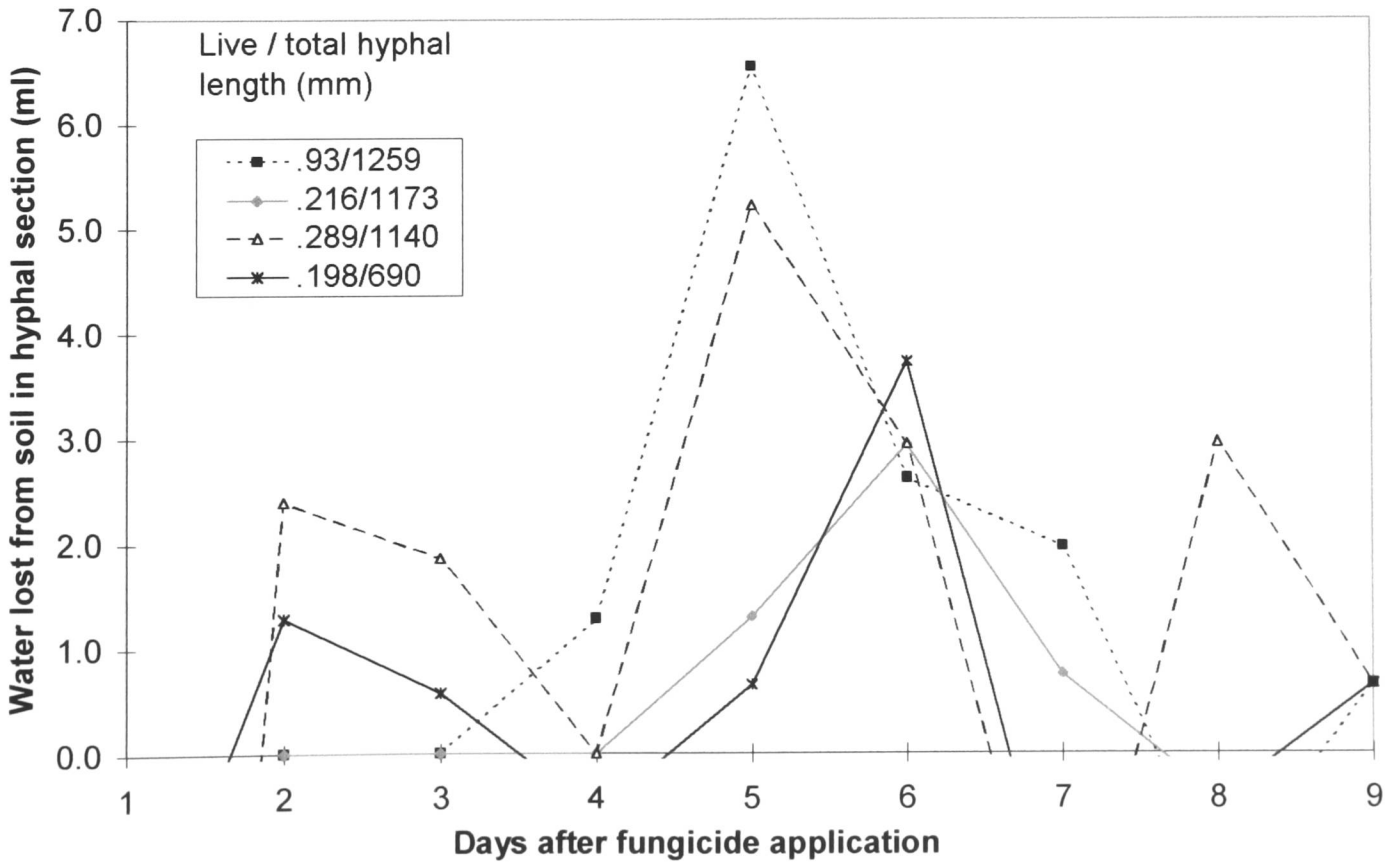
5.3.7 Experiment (C): Chemically reduced functioning of hyphae

The functioning of the hyphae was altered by the application of benomyl fungicide. Hyphae in the hyphal section of the rhizobox were partially destroyed using benomyl fungicide. The destruction was not complete but the vigour of the hyphae was reduced. No data from control, untreated plants is available, since the numbers of plants available had been reduced. The responses in transpiration, photosynthesis, stomatal conductance and water use of plants subjected to benomyl treatment are compared to their responses before treatment.

Removal of water from hyphal section before and after chemical control of hyphae

The daily quantity of water removed from the soil in the hyphal section of the rhizobox after fungicide application is shown in Fig.5.27. In half the replicates, the extraction of water from the hyphal section continued after benomyl application on Day 0 (Fig.5.27). In the other half initially there was no removal of water from the hyphal section and then 4 days after application, water removal occurred. This second group also had the greatest proportional reductions in the length of viable hyphae, seen as the difference between total (viable + nonviable) hyphae, and live hyphae only. The first group had smaller total lengths of hyphae, but also lost less due to fungicide.

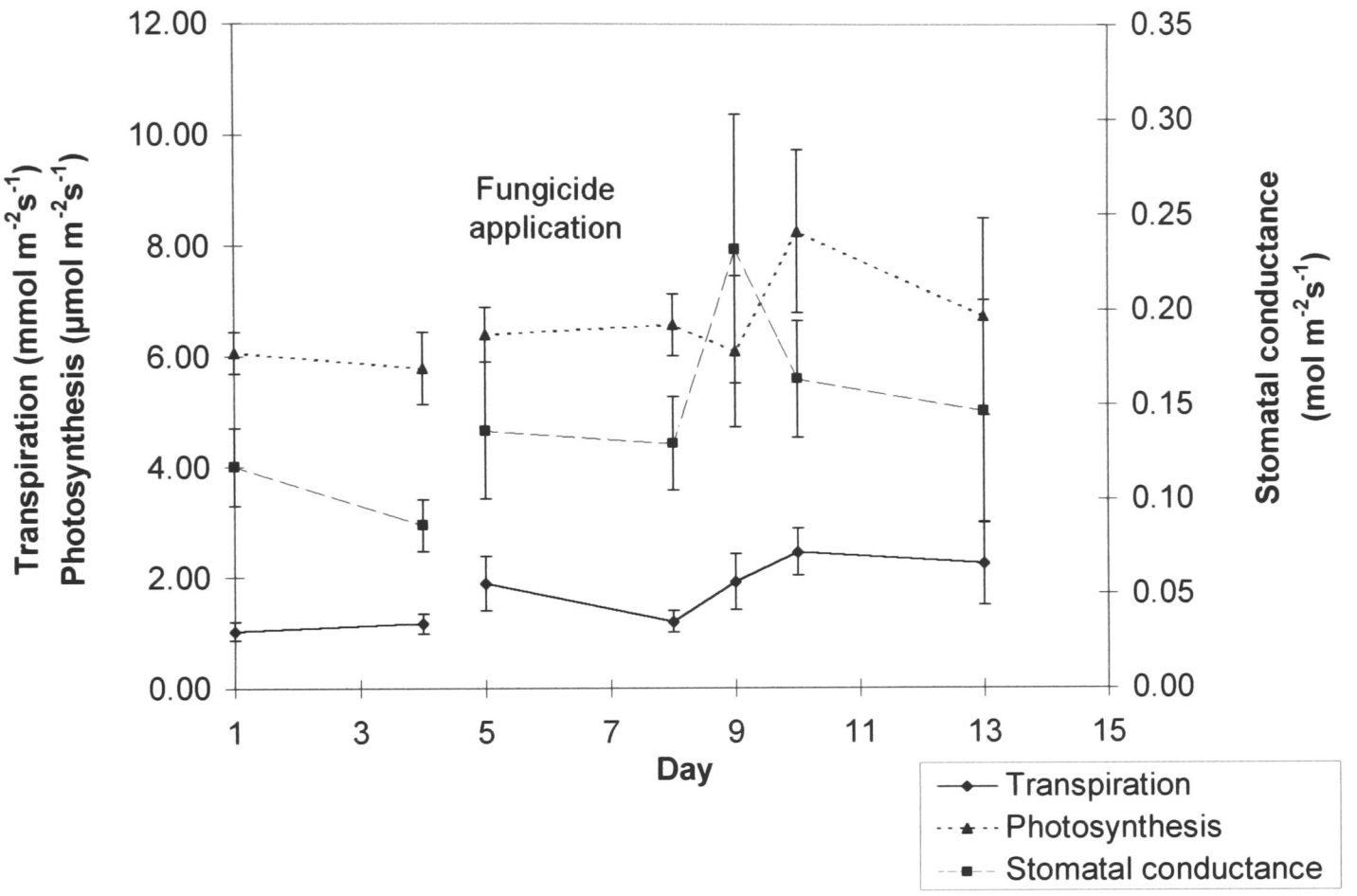
Fig.5.27 Water removal by replicate plants from hyphal section over 10 days after fungicide application



Shoot response

The mean values of leaf processes, stomatal conductance, transpiration and photosynthesis for all plants, are shown over the course of 13 days in Fig.5.28. Fungicide was applied on Day 5. All processes showed a gradual increase over 9 days after application of the fungicide.

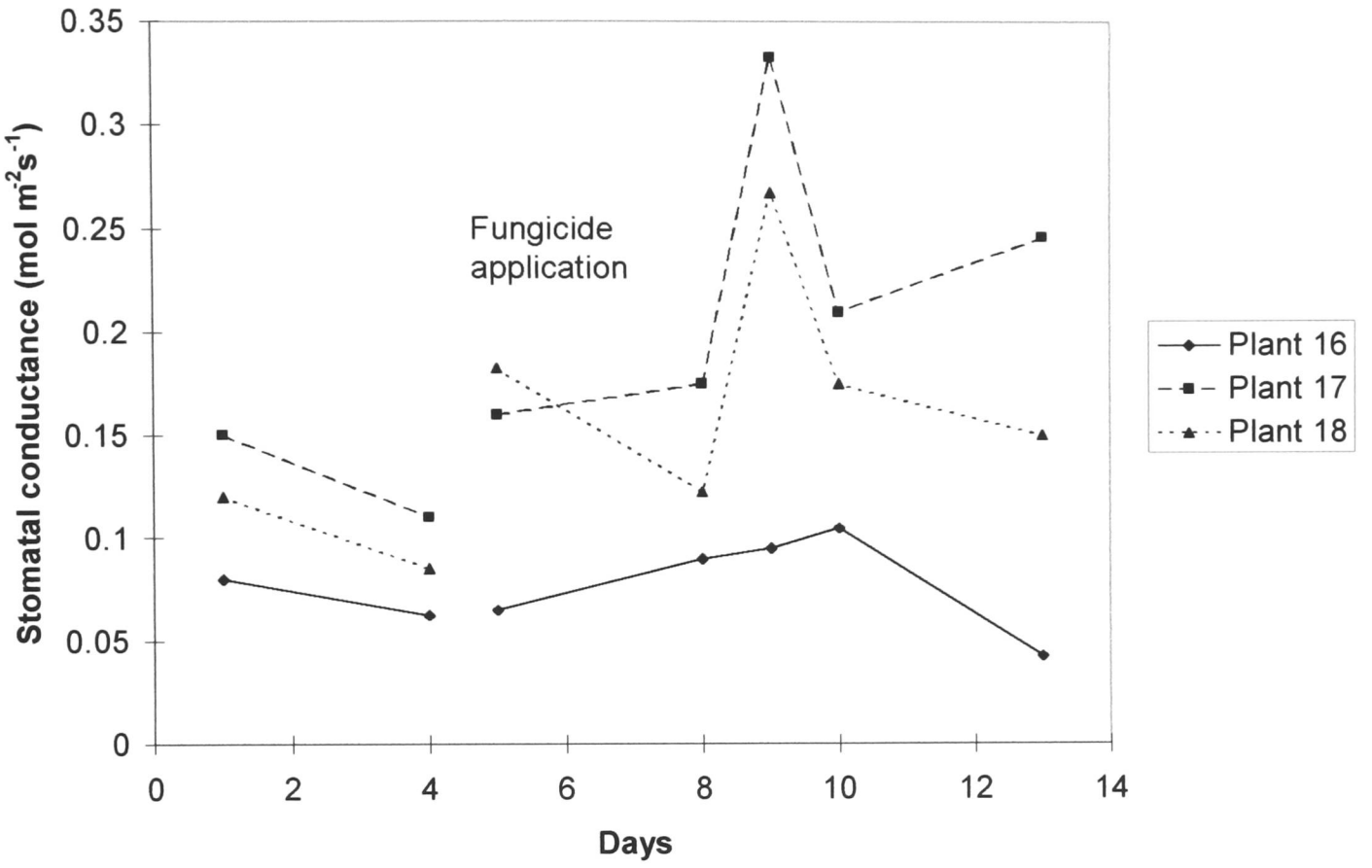
Fig.5.28 Mean leaf processes before and after fungicide application on day 5. Standard error bars are shown.



The individual responses of the replicate plants are shown below to demonstrate their varied responses. Data for the fourth replicate is not shown as the leaves were damaged.

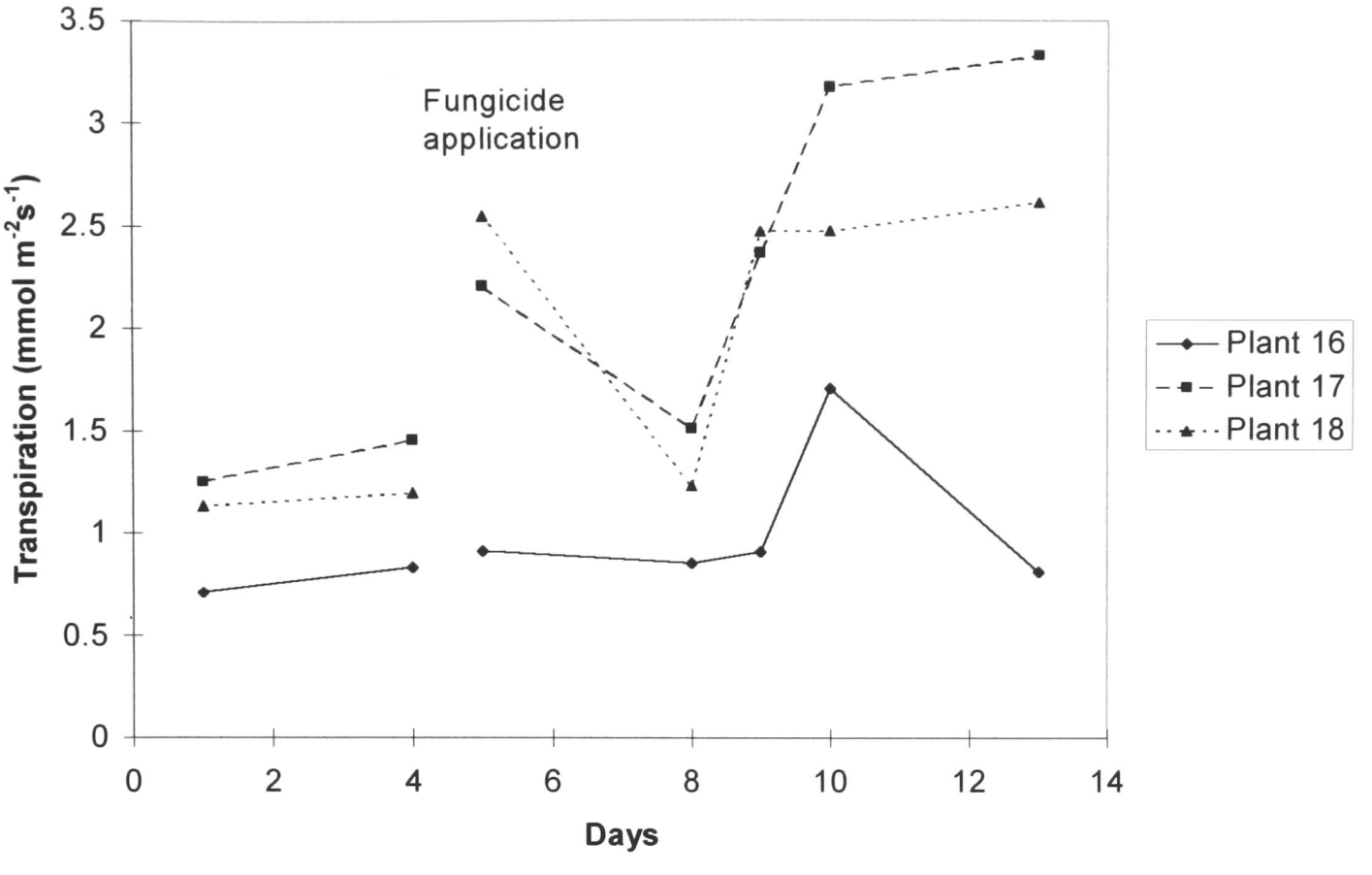
Stomatal conductance increased in all replicates after fungicide application (Fig.5.29) but the increase was not constant. It declined to below its original level in plant 16.

Fig.5.29 Change in stomatal conductance before and after fungicide application showing replicate plants



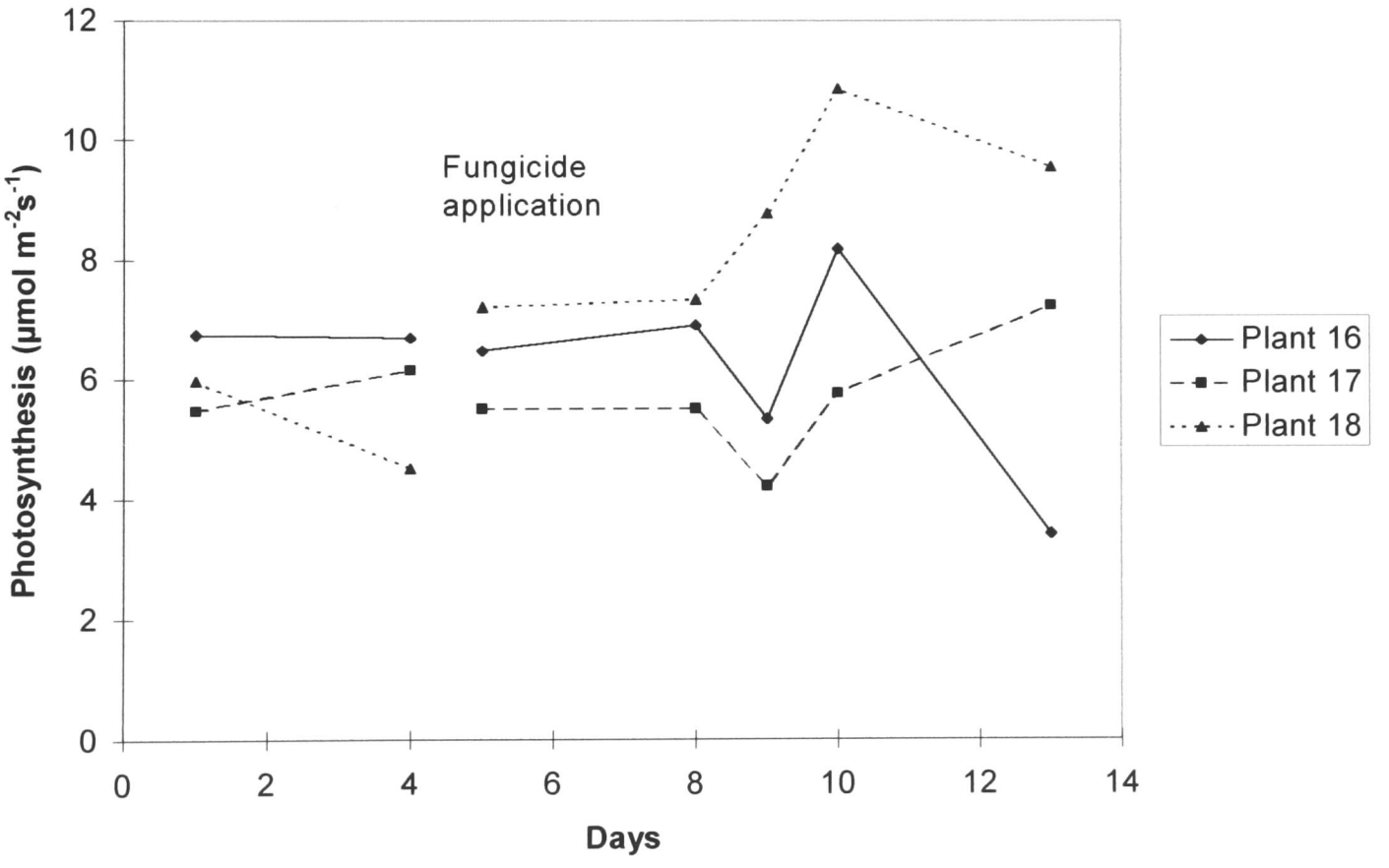
In general transpiration was increased in all replicates after fungicide application (Fig.5.30) but the increase was not constant. It declined to its original level in plant 16.

Fig.5.30 Change in rate of transpiration before and after fungicide application showing replicate plants



Photosynthesis was slightly decreased in 2 plants, but greatly increased in one plant.

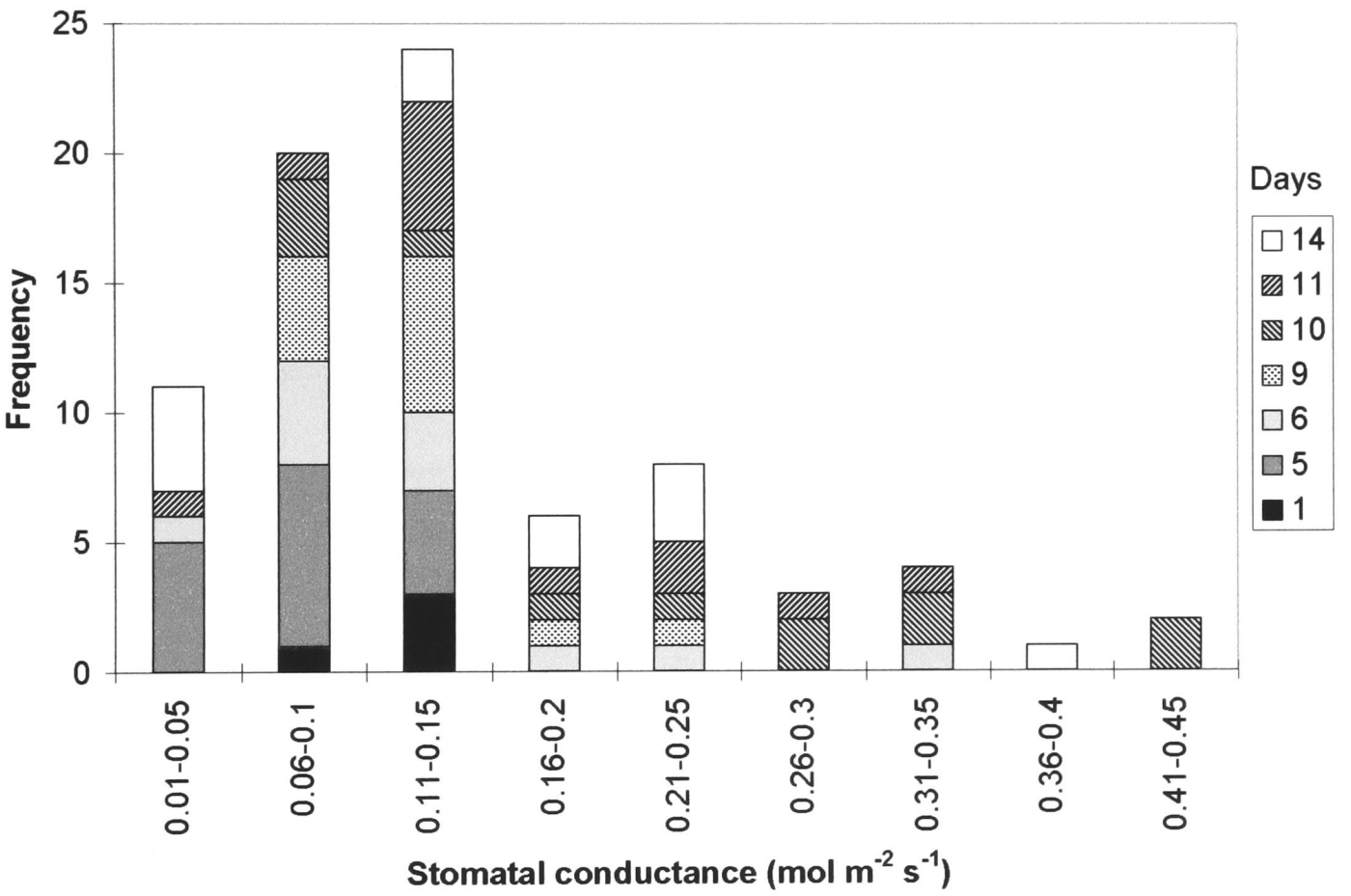
Fig.5.31 Change in rate of photosynthesis before and after fungicide application showing replicate plants



The increase in photosynthesis was in plant 18, which also showed a 15% greater mycorrhizal colonisation than plants 16 and 17, which showed little change in photosynthesis.

The stomatal conductance was examined in greater depth. The values were arranged into groups and plotted in the graph below. A comparison of the range and frequencies of stomatal conductances (Fig.5.32) shows that initially before fungicide application the stomatal conductance was small, below 0.15 mol $m^{-2}s^{-1}$. After fungicide application the stomatal conductance gradually increased in magnitude and in range, with a maximum on Day 10 of 0.45 mol $m^{-2}s^{-1}$. The differences between populations of stomatal conductances on different days was analysed using a Kolmogorov-Smirnov test. This change was significant at the 5% level.

Fig.5.32 Frequency of stomatal conductance before and after fungicide application, on Day 5



The change in leaf processes was compared to the extent of hyphae in the hyphal section (Table 5.10). This was in order to assess whether variations in the shoot responses between plants were caused by differences in the extent of their external hyphae. The total quantity of hyphae, disregarding their metabolic activity were examined first. Then the quantity of viable hyphae were assessed. They were divided into groups of higher or lower than mean length. The plant shoot processes were categorised as showing either an overall increase or decrease after fungicide application. These four possibilities were compared for transpiration, photosynthesis and stomatal conductance.

Table 5.10 Change in leaf processes after fungicide application relative to length of hyphae

Total hyphae

	Long	Short		Long	Short		Long	Short
Transpiration increased	2	1	Stomatal conductance increased	2	1	Photosynthesis increased	1	
Transpiration decreased			Stomatal conductance decreased			Photosynthesis decreased	1	1

Live hyphae

	Long	Short		Long	Short		Long	Short
Transpiration increased	1	2	Stomatal conductance increased	1	2	Photosynthesis increased		1
Transpiration decreased			Stomatal conductance decreased			Photosynthesis decreased	1	1

Long = above mean length
Short = below mean length
Mean total hyphal length 1066 mm/g dry soil
Mean live hyphal length 199mm/g dry soil

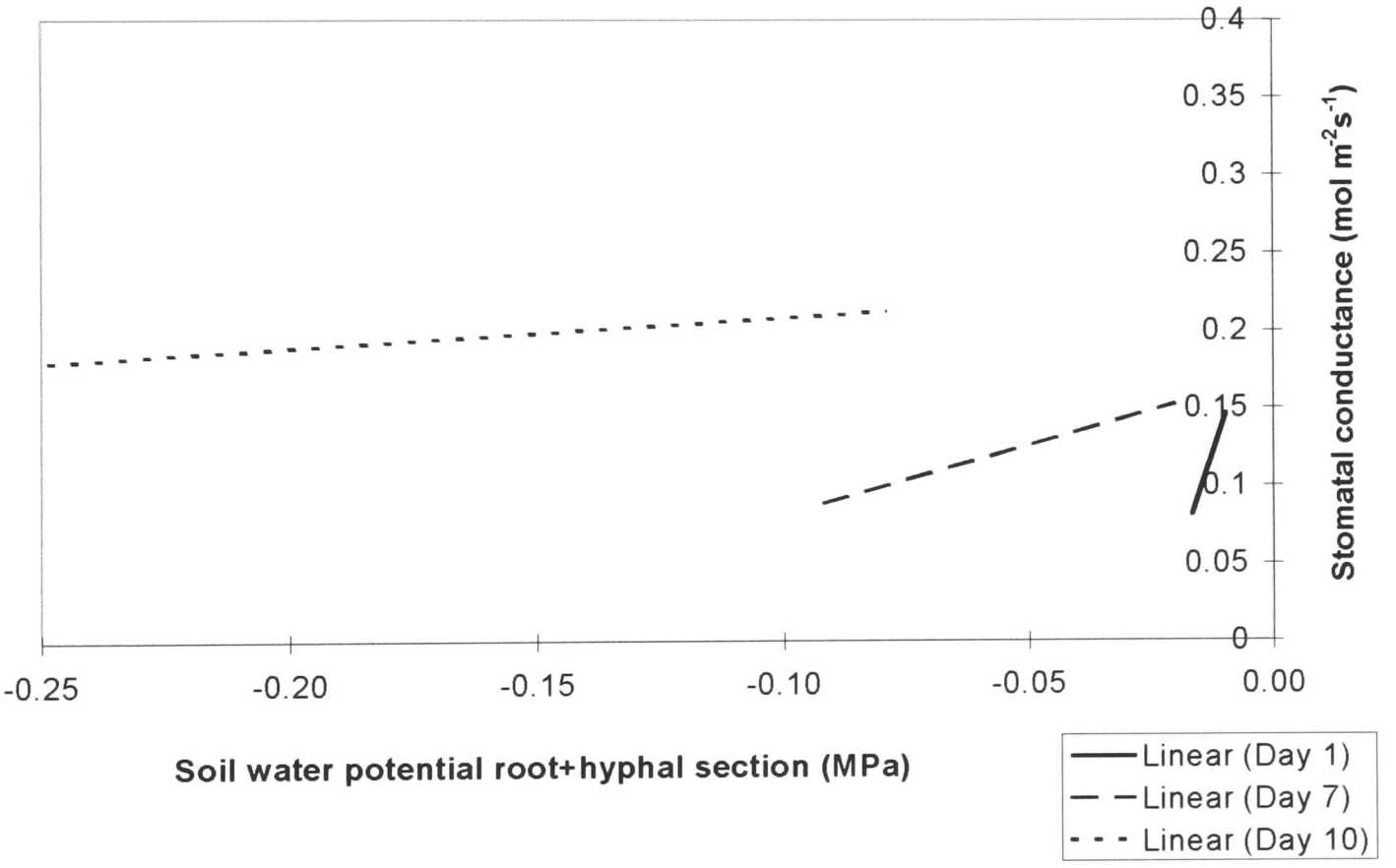
Transpiration and stomatal conductance increased regardless of the extent of total extramatrical hyphae. Photosynthesis decreased in plants both with above or below average length of hyphae. One plant with a higher than mean hyphal length showed an increase in photosynthesis.

Transpiration and stomatal conductance increased regardless of the extent of live hyphae. Photosynthesis again showed an increase or decrease regardless of length of live hyphae.

Stomatal conductance compared to soil and aerial factors

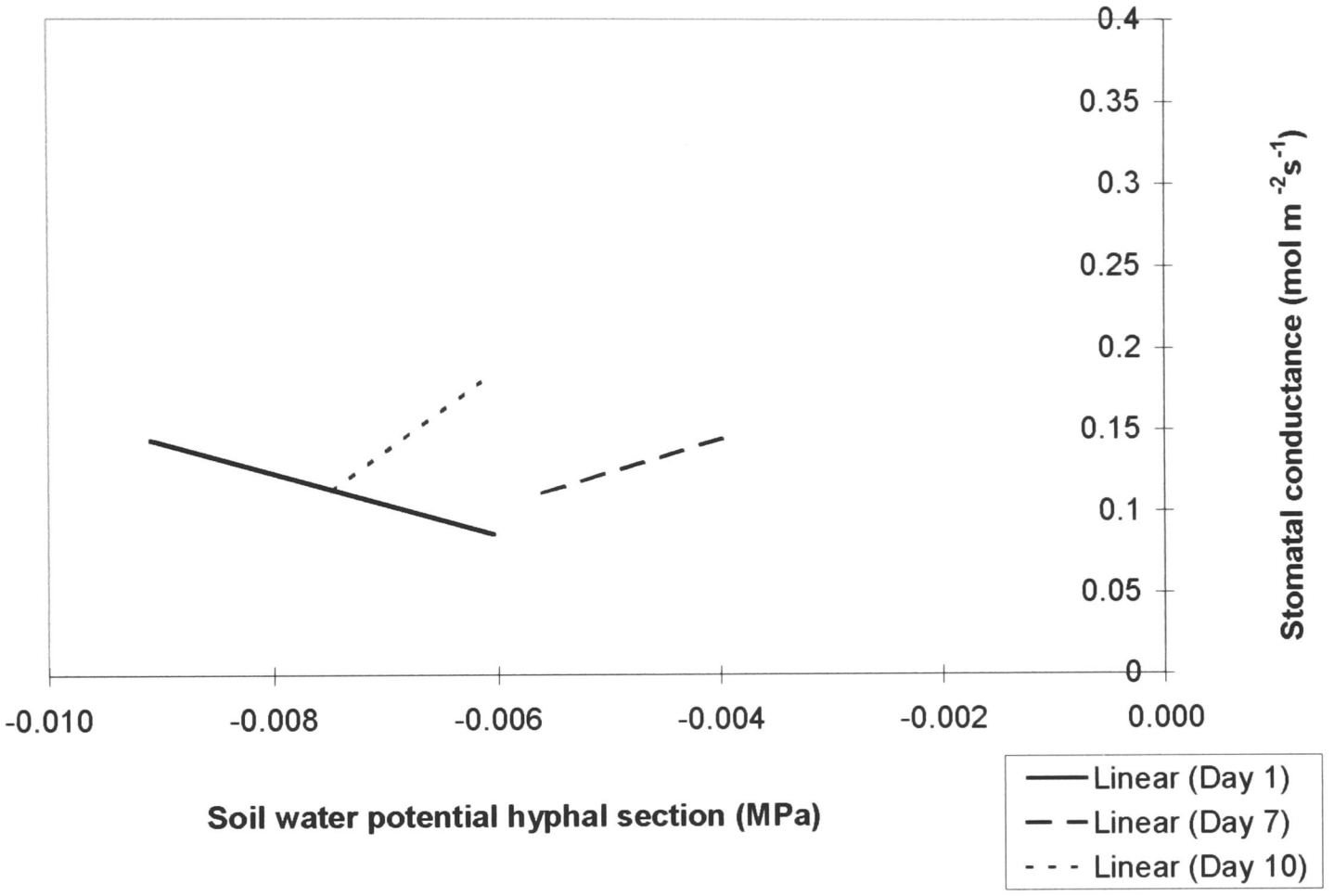
The shoot responses of the plants were compared to the available soil water in each section of the rhizobox. Multiple linear regression was used to express the possible relationship between stomatal conductance and soil water potential in the root+hyphal section, before and after fungicide application. There was a positive overall relationship between stomatal conductance and soil water potential in the root+hyphal section. This relationship is presented graphically in Fig.5.33. There is a change in response of stomatal conductance to soil water potential from extreme to minimal during the course of the experiment, shown by the gradient of the trendlines. However, the only significant relationship was stomatal conductance with soil water potential on Day 10, (co-efficient=0.2, p=0.02).

Fig.5.33 Relationship between stomatal conductance and soil water potential in the root+hyphal section of the rhizobox.



The relationships presented graphically below (Fig.5.34) are between stomatal conductance and soil water potential in the hyphal section of the rhizobox. They show a change from a negative to a positive relationship of stomatal conductance with soil water potential before and after fungicide application. These trendlines, produced by linear regression, were not significant.

Fig.5.34 Relationship between stomatal conductance and soil water potential in the hyphal section of the rhizobox.



5.4 Discussion

The work carried out in these experiments made use of a form of microcosm to examine the role of mycorrhizal hyphae in extending the host plant root system. The microcosm allowed treatment of a portion of the external hyphae independently of the rest of the root-fungal symbiosis. The quantity of water removed from the soil by these hyphae could be measured. In addition they could be severed from the rest of the root-fungal system or their functioning impaired by the use of a fungicide. The plant response to these treatments could be used as a measure of the significance to the whole plant of these hyphae.

5.4.1 Limitations of the rhizobox method

In this study 20 rhizoboxes were constructed. This was largely because of the cost of materials, glass dishes cut to specification and very fine nylon mesh, and thus the cost of individual units. Steel mesh was also needed to ensure that roots could not penetrate the hyphal section. It was particularly important to avoid this as the study was over a long period, during which time the plant roots would become woody, and more likely to penetrate the nylon mesh. The problem of roots escaping the root+hyphal section occurred in the study of Faber *et al.* (1991) and may have confused some of their results. The steel mesh was successful in controlling the spread of root growth. The woody root system could be seen, at harvest, to have spread out to the edges of the rhizobox, and the volume of soil in the root+hyphal section was then filled with finer, non-woody roots (Fig.5.4). It was necessary to have a root+hyphal section which was large enough to support the plant over an extended period. The hyphal section had to be small enough so that a change in the soil water content would be measurable. Therefore the relative sizes of the two section was important.

The rhizoboxes used here were constructed so that the root+hyphal section was above the hyphal section, rather than adjacent to each other as in the studies of Faber *et al.* (1991) and George *et al.* (1992). This design was successfully employed in investigations of the water transport of ectomycorrhiza (Duddridge *et al.* 1980). It was chosen to minimise evaporation from the soil in the hyphal section by sealing it

permanently to the upper root+hyphal section. The only joint in this rhizobox was between the two sections. In previous published studies, the rhizoboxes were rectangular with many sealed edges. These edges were all potential sources of losses of water or water vapour. Evaporation over short periods was effectively minimised in this study, although over many weeks there were losses of water from some rhizobox hyphal sections, possibly through the watering inlet. More sensitive soil water monitoring devices would possibly have shown evaporative losses. George *et al.* (1992) were able to construct microtensiometers 1.2 mm in diameter which were monitored using a datalogger, and so produced detailed measurements of soil water losses. A disadvantage of the rhizobox sections being stacked one above the other was that the control of soil water content was more difficult than in previous systems. Evaporation or hyphal water use were the only means of achieving very dry conditions in the hyphal section. This was achieved in some rhizoboxes. It would be useful in further work to more strictly control the water contents at given levels. It had been initially intended to maintain conditions of continual adequate soil water or water deficit in each rhizobox section, in varying combinations, to study long-term influences over water availability in the two sections. However this degree of control was not achieved.

The rhizobox was used as a method for generating a "split-root" situation between hyphae and roots. Non-mycorrhizal plants were not required in this set of experiments, because it was the role of hyphae themselves at varying levels of water availability which was of interest. In addition non-mycorrhizal plants would not have been of the same size, or nutrient status as mycorrhizal plants, which would have altered their response to soil drying. Control plants would have been mycorrhizal but with no imposed treatment of hyphal severing or chemical control. However because of the loss of some plants, the response in terms of water use and gaseous exchange of treated plants was compared with that before treatment. Clearly future work would require the use of more replicate plants.

The rhizobox system was advantageous in that it allowed the measurement of a separate portion of the fungal mycelium. Conventional pot studies generally measure the responses the root-fungal association as a whole. They do not differentiate

between host or fungal response, nor between internal or external structures of the fungus. The rhizobox allows some differentiation in the responses of parts of the association to imposed treatments.

The rhizobox is disadvantageous because of its restriction of the plant root system. This restriction is imposed by the need to encourage root growth near a mesh barrier through which external hyphae may pass. The time required to achieve growth of the hyphae through the mesh extends the duration of the experiment. During this time the root restriction reduces the vigour of the plants. The relative volume of the root section and the hyphal section may affect the relative contribution of roots and hyphae to responses to the soil environment. Over long periods with restricted root growth, the growth of plants of one treatment may catch up with those of another treatment. In this case however there were no long-term treatment differences between plants.

5.4.2 Plant response to growth in the rhizobox

The plants were assessed in terms of their shoot responses to environmental conditions. They appeared to be responding normally to changes in the environment, as compared with other studies where plants were not grown in the restricting environment of a rhizobox. For example, transpiration is generally lowered at reduced soil water potential during drying cycles (Kaufmann 1968). This is caused by increased leaf diffusion resistance and often the loss of some leaves during drying. A range of species was compared, and showed that transpiration either remained lower after rewatering or recovered completely depending on the species examined. The water use and transpiration of a range of species was also compared to soil water availability in some other studies (Jarvis 1979, Jarvis and Jarvis 1963i,ii). Water use on a leaf area basis and transpiration followed a nearly linear relationship with soil water potential. In this study there was some variation about the linear relationship, but a similar trend was seen.

It was shown in this project that carbon dioxide uptake was closely related to leaf stomatal conductance. The relationship between photosynthesis and stomatal conductance followed a hyperbolic curve, similar to the results of Jarvis and Jarvis

(1979). Stomatal conductance was dependent on a number of environmental factors, including light and temperature (Schulze and Hall 1982). There was a slight decrease in stomatal conductance with increasing air temperature which has been previously shown (Wuenscher and Kozlowski 1971). Stomatal conductance was seen to decrease at high light intensity. This is in contrast to other studies and is likely to be due to interactions between light intensity and temperature (Pereira and Kozlowski 1977). Stomata usually open in the light but it is not clear whether this is a direct effect of light or because photosynthesis decreases the internal concentration of CO_2. The response of plants to increasing irradiance may also vary with plant species, such as shown in *Impatiens pallida,* where stomatal conductance increased initially and then decreased after several hours (Schulz *et al.* 1993).

During experimentation transpiration is also affected by leaf area. Plants have shown alterations in size and leaf area when grown in microcosms (Faber *et al.* 1991). Leaf size was also reduced in this study but was probably an effect of repeated drought stress.

5.4.3 Comparison of rhizobox studies

There was high variation in gas exchange between plants in these experiments. It was attempted to explain this variation in terms soil water content, mycorrhizal colonisation and external hyphal length. No clear relationships were found but there were some trends. As expected gas exchange was limited by reduced soil water content, during drying cycles. Soil water content was independently maintained at similar levels for all plants so that they experienced similar water deficit stress despite their variation in size. Water loss from the volume of soil containing hyphae was also seen during drying cycles. The difference in water loss from these sections was compared to the length of external hyphae found in the sections and found to be broadly related. In my study comparison in gas exchange was made before and after severing of hyphae. There was no comparison with rhizoboxes with unsevered hyphae. The response to severing of hyphae was variable. In some plants transpiration was lower after hyphal severing. This is a similar result as Faber *et al.* (1991). However in that study plants where hyphae were severed were compared with intact

plants. Intact plants transpired 35% more water than plants with severed hyphae over a 16 hour period, as determined by loss of water from the hyphal section. The source was thought to be the hyphal chamber, suggested by a RbCl tracer and lower soil moisture content in the outer chamber. In this study, the change in transpiration was measured directly, immediately before and after severing of the hyphae. Contradictory results were reported by George *et al.* (1992). They found no difference in water loss from hyphal compartments measured by tensiometers under well-watered and water-stress conditions whether hyphae were present or not. Severance of hyphae did not affect water loss from hyphal compartments.

The relative sizes of rhizoboxes used in this and previous studies were very different. The volumes of soil in each section in these studies and this one are shown below.

Table 5.10 relative sizes of rhizoboxes

Soil volume (cm^3) in:	Root+hyphal section	Hyphal section
Kothari *et al.* (1990)	3000	3000
Faber *et al.* (1991)	214	479
George *et al.*(1992)	128	320
Current study	1655	345

The hyphal section in this study may have been too small to be of significant importance relative to the quantity of hyphae in the root+hyphal section. The quantity of hyphae in the root+hyphal section was not measured because of the laborious nature of the extraction and the much higher likelihood of contamination by other fungi. Consequently it was not known what proportion of the total external hyphal mycelium was in the hyphal section of the rhizobox.

The difference in rhizobox dimensions possibly accounts for the difference in the conclusions of previous studies. In addition they were concerned with different host-fungal associations. Differing responses of fungal symbionts have been shown to imposed treatments (Ruizlozano and Azcon 1995), who also used a rhizobox where hyphae were separated from lettuce roots by mesh. Water was supplied to the hyphal compartment at three levels of watering. Mycorrhizal plants had higher water and nutrient contents. In addition different abilities of specific mycelia of *Glomus*

deserticola and *Glomus fasciculatum* were also expressed in terms of nutritional and leaf gas-exchange parameters.

5.4.4 Chemical control of fungal hyphae

The fungicide benomyl [methyl 1-(butylcarbamoyl)benzimidazol-2-ylcarbamate] was applied to eliminate the hyphae in the hyphal section of the rhizobox. Merryweather and Fitter (1996) showed that in field studies benomyl was particularly useful for controlling AMF in field studies where the root system was restricted. This may have been because the fungicide was not washed away down the soil profile. In this study the fungicide was applied to a confined volume of soil and was likely to reach all the hyphae in this volume. Complete control was not achieved but the functioning of the hyphae was reduced.

A possible complication in the use of fungicide may have arisen. AMF are aseptate, that is, their hyphae have no regularly occurring cross-walls along their length. The fungicide could be transported into the hyphae in the root+hyphae section of the rhizobox, and possibly into the plant also. Benomyl does not show any phytotoxic effects, but the effect on hyphae in the root+hyphal section could alter the plant response. It may be that this contributed to the lack of a clear change in plant gas exchange, after fungicide application.

It has been shown to reduce metabolically active fungi of *G.intraradices* and *G.caledonium* in onion roots, 3 days after application (Kough *et al.* 1987). This was a similar time period as this study. AMF colonisation was depressed 2 weeks after application (Kough *et al.* 1987, Fitter and Nichols 1988). During the time of this experiment, viable fungi could have been expected to be destroyed.

Mechanism

Benomyl fungicide has been used in a number of studies to selectively remove fungi from soil and to examine phosphate metabolism in fungi. The chemical inhibits hyphal growth (Carr and Hinkley1985) but does not kill spores or hyphae in excised root pieces. The reduced functioning of the AMF was examined using a rhizobox with a hyphal section by Larsen *et al.* (1996). P transport was inhibited in *Glomus*

caledonium colonising cucumber roots when benomyl was added to the hyphal compartment in the root+hyphal compartment. The fungal alkaline phosphatase activity was unaffected. The reduced P inflow into host plant roots was also shown by Fitter and Nichols (1988) although there was no effect on P concentration in the soil. Sukarno *et al.* (1996) showed a reduced transfer of P across the plant-fungus interface. The rate of P uptake by living fungi was not affected but development of living external hyphae in soil was reduced so the total P supplied by fungus reduced. Benomyl is believed to act partly on the fungal cytoskeleton. Transfer of phosphate is likely to involve cytoplasmic streaming (Harley and Smith 1983) which would be affected by changes in the cytoskeleton.

Although there is active transport of P through hyphae, which is affected by benomyl application, there may be no effect on other potential drought signalling compounds. This study showed no clear evidence of any alteration in the plant-fungal symbiosis, in terms of their water relations.

5.4.5 Extraction of hyphae

The possible methods of extraction of hyphae were compared by Green *et al.* (1994) These were membrane filtration, as used in this study (Hanssen 1974, Jakobsen *et al.*1992), mycelium collection on rotating wires (Vilarino *et al.* 1993), and sucrose flotation (Schubert *et al.* 1987, Hamel *et al.* 1990). Membrane filtration was the most effective at extracting mycelium at high hyphal densities. There were no differences between methods at low densities. Some of the errors incurred in the membrane filter technique were examined by Sundman and Sivela (1978). The highest error was incurred in the soil sampling, due to the heterogeneity of soil. There was also greater error if the soil solution was concentrated. They recommended soil dilutions of 10^{-3} . The soil in the rhizoboxes was sieved and homogenised in solution before fungal assessment. This reduced the error in sampling in the current study. Large pieces of debris were removed by coarse sieving, and soil particles were broken up with a dispersant. These procedures minimised the quantity of soil collected on the filter membrane, and permitted measurement of hyphae more easily.

5.4.6 Extraradical hyphae

The extraradical spreading of *Glomus intraradices* in tomato was described by Bago *et al.* (1998). First there is proliferation of runner hyphae acting as conducting channels, which divide dichotomously and extend the fungal colony radially, second there is development of arbuscle-like structures, which are formed at regular intervals along the runner hyphae, and finally formation of spores in zones already colonised by runner hyphae and arbuscle-like structures. This study showed the intricate architecture of extraradical hyphae.

The mass of extraradical hyphae is usually linearly correlated with the degree of mycorrhizal infection (Sanders *et al.*1977) However this was not found in this study, where the relationship was poor between these parameters. Length of external hyphae varied from 0 to 1 m g^{-1} dry soil, or 0 to 0.64 m cm^{-3}, in this study. This can be compared to a mean hyphal density of 3 m cm^{-3} (George *et al.*1992), 0.35 m cm^{-3} (Faber *et al.* 1991), and 0.07 m cm^{-3} (Green *et al.*1994) for various species of AMF also grown in rhizoboxes and extracted from the hyphal section.

Table 5.11 Length of external hyphae per unit volume of soil 5-7wks after inoculation. Length of non-AMF hyphae from uninoculated soil were subtracted. Data is from a range of substrates, and seasons.

Fungus	Length (m cm^{-3} soil)	Reference
Grown in pots		
A.laevis	10.6	Abbott & Robson 1985
G.fasciculatum	2.5	Abbott & Robson 1985
G.calospora	12.3	Abbott & Robson 1985
G.tenue	14.2	Abbott & Robson 1985
G.clarum	0.22-0.27	Schubert *et al.* 1987
Grown in hyphal chambers of rhizoboxes		
G.caledonium	3.1	Larsen *et al.* 1996
G.geosporum	0.07-0.1	Green *et al.*1994
G.monosporum	0.23	Green *et al.* 1994
G.manihotis	0.02-0.07	Green *et al.* 1994
G.mosseae	3	George *et al.* 1992
G.clairodeum	0.35	Faber *et al.* 1991
G.intraradices	0-1	current study
Extracted from field soil		
Mixed species	81-111	Miller *et al.* 1995

Specific root length and root mass have been shown to have a strong relationship with external hyphal length (Miller *et al.* 1995), but this was not seen in the current study. In their study, during drought specific root length was reduced but not external hyphal length. Recovery occurred by increasing external hyphal length not specific root length. This meant that growth was co-ordinated between root morphology and AMF hyphae.

The length of external mycorrhizal fungi was calculated from the total length of fungal hyphae after subtracting a base level of hyphae which were taken to be other contaminating species. This was necessary because hyphae from different species could not be differentiated, so it could not be assumed that all hyphae present were those of the mycorrhizal fungus. There have been many studies showing a negative effect of mycorrhizal fungi on other plant parasitic soil fungi. However there has been little work on their influence on non-parasitic fungi. It is not clear from this study whether there was a decrease in the incidence of other soil fungi due to the presence of the mycorrhizal fungi. Consequently, it could not be determined whether the subtraction of a base level of fungi was necessary. In terms of the relationship between external hyphae and other mycorrhizal characteristics, this is not important. It is only relevant where estimates of external hyphal length are compared between habitats or experimental work. In field studies, where mixed species are considered, all collected hyphae are measured. However in other pot studies, where one species in often being examined, some allowance is made for potential contaminating species.

5.4.7 Vital staining

The formation of coloured formazan products by the reduction of tetrazolium salts has been previously used as an indicator of biological reducing systems. Tetrazolium compounds have been used as vital stains for a number of mycorrhizal tissues, such as the plant-fungus interface (Smith *et al.* 1994), fungal spores (Walley and Germida 1995, Meier and Charvat 1993, McGee *et al.* 1997), mycorrhizal colonisation of roots and intraradical hyphae (Smith and Dickson 1991, Bentivenga and Hetrick 1991, 1992, Schaffer and Peterson 1993, Kough *et al.* 1987), and external mycelium (Saito *et al.* 1993).

There are few estimates of the length of live AMF hyphae, shown in Table 5.12. The calculated length of live hyphae in the hyphal section of the rhizobox varied between 0.088-0.448 m g^{-1} dry soil, or 0.06-0.29 m cm^{-3}, given a soil bulk density of 0.64 g cm^{-3}. That is approximately 9 to 45% of the maximum amount of external hyphae found in the current study, that were active. A number of different methods have been suggested to facilitate assessments of living fungal structures, such as fluorescein diacetate (FDA) (Ingham and Klein 1984, Soderstrom 1977), differential fluorescent stain (Morris *et al.* 1997), immunofluorescence assay (Kough and Linderman 1986), or tetrazolium salts. These are dehydrogenase active stains. They assess different reactions within the living tissue but are all used as measures of tissue viability. Two tetrazolium compounds and fluorescein diacetate have been compared in the assessment of external mycorrhizal hyphae and root colonisation (Hamel *et al.* 1990). The assessments of hyphal length were made microscopically using a gridline intersect method. The lowest standard error was achieved in the measurements using fluorescein diacetate. However a grid with more intersections was used in the FDA assessment, and a different magnification was used. It has been suggested (Kough *et al.* 1987) that tetrazolium staining was unsuitable for use with external hyphae because of poor contrast between soil particles and purple formazan. However this was not found in this study because of the use of a soil dispersant to break up larger soil particles, and careful filtering of the soil solution. Use of the same microscope for both total and live hyphal measurements, reduced the possibility of differences in variance between estimates.

Table 5.12 Length of viable external hyphae per unit volume of soil.

Viable hyphae	m cm^{-3} soil	Reference
Pot study		
G.clarum	0.01-0.12	Schubert *et al.* 1987
G.intraradices	0.06-0.29	current study
Field soil sample		
Mixed species	0.24-1.13	Morris *et al.* 1997
Mixed species	0.5	McGee *et al.* 1997

After severing, hyphae could die back within the root+hyphal section. However a hyphal network can survive for some months in soil separated from a root

system (McGee *et al.* 1997). This suggests that within the time period during which measurements were taken, the active hyphal length would be little reduced.

Buffering

The use of a buffer in the staining solution was tested by Meier and Charvat (1993). Higher viability estimates of fresh spores were shown using the buffered method compared to unbuffered for the same incubation period of 4 hours. However autoclaved spores were also stained using the buffered method and there was no significant difference in viability between killed and non-killed spores. The viability estimate in the unbuffered method continued to rise for 70 hours. If the estimates of viability are compared before staining of buffered control spores occurred, higher percentages were found using the unbuffered solution. Unbuffered solution required a longer incubation time but appeared to give a reliable estimate of viability. Unbuffered solutions were also used in the present study.

Incubation

Clearly incubation time is an important consideration in the development of the tetrazolium stain. Overnight staining was found to be suitable, noted by assessing the stained hyphal length at intervals, described earlier. Temperatures ranged from room temperature (Hamel *et al.* 1990) to 36°C in various methods (Meier and Charvat 1993). A constant temperature of 20°C was chosen for this study, similar to the unbuffered solution mentioned above.

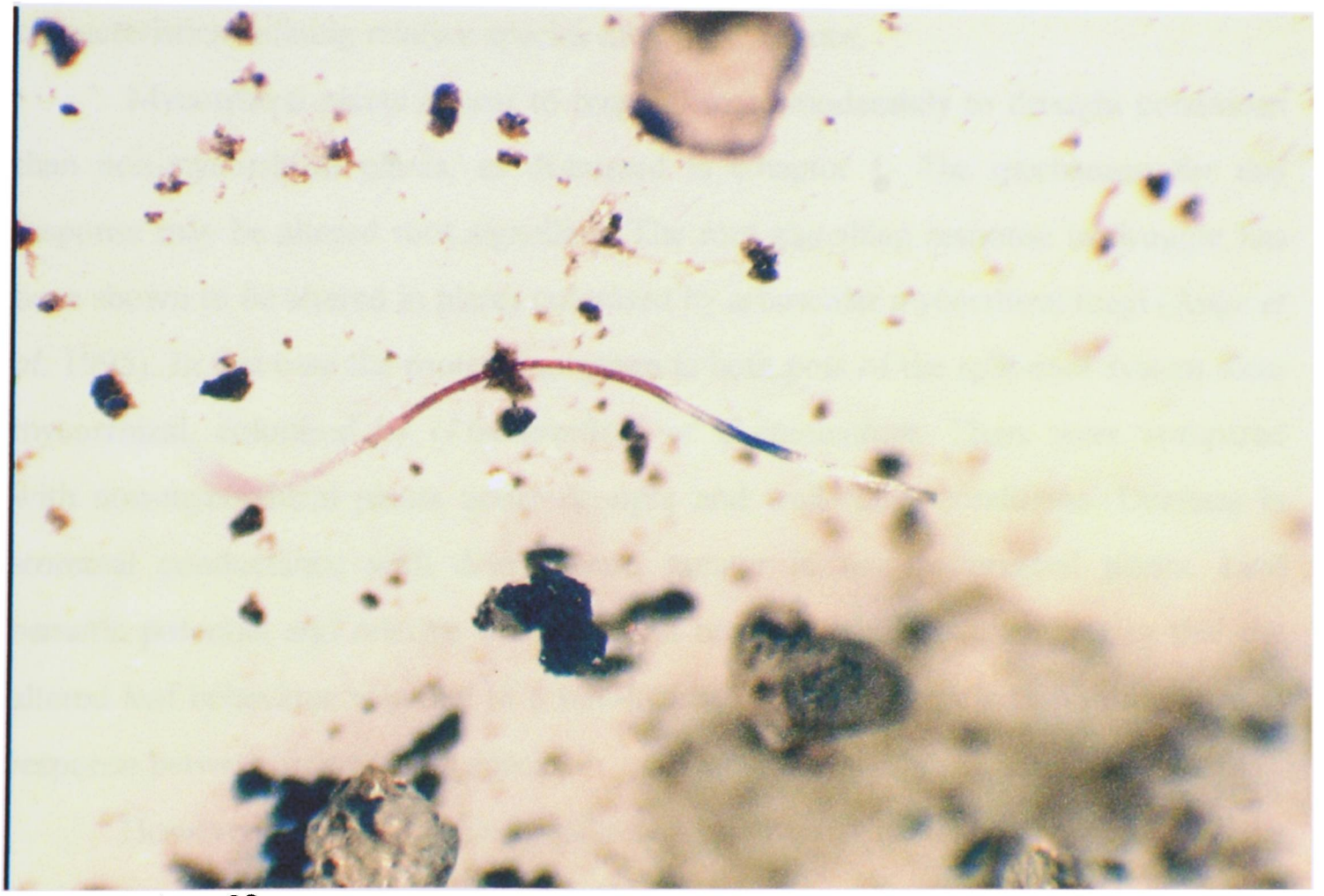
In the experiments of Meier and Charvat (1993), the colour reaction was not stable, so the percentage of red-stained spores varied by as much as 20% depending on time. Colour was found to be stable in this study, the same hyphal length was found 24 hours after the initial assessment. This was adequate, since samples were analysed within 8 hours of extraction.

The concentration of the tetrazolium salt was 0.1%w/v, the same as the present study, in all published work.

Variation in colour

A positive staining reaction with tetrazolium compounds can yield up to three types of coloured formazan products of red, purple and blue crystals (Altman 1974). This characteristic was also noted during the present study, as shown in Fig.5.30. Formation of the different colours depends partly on the varying concentrations of chellating ions such as copper, silver, cobalt and nickel and on the polarity of the solvent but never indicates a negative reaction. Altman (1974) suggested that different colours were not due to intermediate reduction products but were different forms of the same formazan due to chelation. As a result of this variability cobalt ions are routinely added to tetrazolium solutions to stabilise formazan formation to the blue colour. In this study this was not done, to simplify the staining solution, and all colours were noted as positive results. Only as absence of colour was noted as a negative result, as an indication of a lack of dehydrogenase reaction.

Fig.5.30 Fungal hypha showing blue and pink colour achieved with vital staining using tetrazolium chloride



Magnification x80

5.4.8 Signalling

If plants show changes in leaf gas exchange or growth due solely to soil water potential, this is said to be a hydraulic response. If this is not the case and there are changes in the leaf response before any changes in leaf water potential, there is said to be a non-hydraulic response in the leaf, or root signalling. Root signalling was demonstrated in six temperate non-mycorrhizal tree species by Croker *et al.*(1998). Roots were divided between two pots. One pot experienced either root drying, between soil water potentials of -0.01 and -0.1 MPa, or root severing. Drying half the root system caused non-hydraulic declines in stomatal conductance in all species. This was demonstrated because leaf osmotic potentials were similar among drying treatments, whereas the stomatal conductance was closely related to soil matric potential. Hence the stomatal sensitivity was linked to a non-hydraulic root-sourced signal rather than a direct response to water stress within the leaf. Stomatal sensitivity was not closely correlated with previously identified lethal leaf water potentials or their capacity for osmotic adjustment. This suggested that stomatal sensitivity to non-hydraulic root signals may be mechanistically linked to a limited extent with other characteristics defining relative species drought tolerance.

Mycorrhizal plants appear to respond more moderately to drought conditions than non-mycorrhizal plants, as discussed in Chapter 1. The mechanism for this response may be altered root signalling. The root signalling response to drought has been shown to be altered in plants colonised by arbuscular mycorrhizal fungi (Auge *et al.* 1995). In this case the roots of sorghum in both pots of the split-root system were mycorrhizal, colonised by *G.intraradices* or *G.etunicatum*. These were compared with non-mycorrhizal plants under drought and well-water conditions. Declines in stomatal conductance with drying were greater in non-mycorrhizal plants. Leaf osmotic potential and relative water content remained the same, suggesting that the altered leaf behaviour was due to a non-hydraulic factor. There was no difference in response between mycorrhizal species.

However the advantageous influence of mycorrhiza under drying conditions was not seen in a cowpea-Glomus *intraradices* association (Ebel *et al.* 1996). Stomatal conductance in mycorrhizal plants was reduced to a greater degree and on

more days than non-mycorrhizal plants. This was attributed to quicker soil drying in mycorrhizal pots. If the rate of soil drying was influenced by the mycorrhizal colonisation, it may be that the quantity of external hyphae had a role to play in the plant response to drying.

These experiments were similar to those described above in that they used a the concept of split-root experiments to investigate whole plant responses to drying. However rather than comparing mycorrhizal root systems with non-mycorrhizal root systems, only mycorrhizal plants were tested. Also these were split-hyphae systems, in that the whole plant response to hyphae, rather than roots, was compared.

The difference between mycorrhizal plants and non-mycorrhizal plants in root signalling, lies in the difference of their rate of decrease in stomatal conductivity under drought conditions, which is higher in non-mycorrhizal plants. If hyphae were acting as an extension of the root system, the rate of decline might be expected to be altered before and after hyphae were severed from the rest of the root system. There was a large variation in the gas exchange responses between plants, particularly after the severing treatment. These changes in leaf responses could not be attributed solely to the soil water potential. There was found to be a change in response of the plant gas exchange to soil water potential surrounding the external hyphae, after hyphae were severed. This alteration in the slope of the stomatal conductance response after severing would appear to suggest that the plants were initially responding to signals from the hyphae of greater water availability in the hyphal section. After severing the plant no longer responded at the same rate to the difference in soil water potential. Stomatal conductance, transpiration and photosynthesis were generally reduced. Nor did they immediately recover to previous levels. There was a gradual reduction in the relationship between shoot processes and the soil water potential in the hyphal section of the rhizobox.

After chemical treatment of the external mycelium, there was a trend of increasing stomatal conductance, transpiration and photosynthesis. There was no clear trend in the relationship between shoot processes and soil water potential in the hyphal section of the rhizobox. This experiment was carried out over a longer period than that above where hyphae were severed. The length of time over which

measurements were taken appeared to have a bearing on the relationship between shoot processes and soil water potential. Over periods of a few hours stomatal conductance responded to soil water potential in the hyphal section, whereas when periods of days or weeks were considered there was no significant relationship between these parameters.

If the external mycelium played no part in the water relations of the plant, it would be expected that there would be no change in the relationship between shoot processes and soil water potential, despite changes in the quantity or functioning of the mycelium. However this was not the case. The plant did show changes in its shoot response with changes in the external mycelium. A change in stomatal conductance was shown, when treatments imposed on the external hyphae altered their quantity or metabolic activity. Although there were not sufficient replicate plants to demonstrate significant differences, there were indications that the plants were able to respond as a whole system to changes in the external portion of the fungal partner. This suggests some form of signalling between the two. This is particularly true since the response was over a short time period, before any changes in plant nutrient status could be expected to have occurred. The plant response was also related to soil water availability on a short term basis, indicated by changes in the stomatal conductance with soil water potential, after treatment of the external fungal hyphae. These results suggested that a relationship existed between the host plant, the fungus and water availability.

Sensitivity of non-hydraulic signal

It was mentioned above that the relative sizes of the rhizobox sections could influence the effect of mycorrhizal hyphae on plant water relations, by determining the length of external hyphae. This implies a quantitative response in gas exchange to the hyphal length. The relative quantities of hyphae in a dry or a wet section of the rhizobox then become important. This idea of sensitivity in the signalling response was tested in mycorrhizal root systems by Ebel *et al.* (1994) in a sorghum and *G.intraradices* association. The mycorrhizal root system was split into four pots. The degree of drying was varied by imposing drought in one, two or three pots to examine

the degree of sensitivity to a non-hydraulic drought signal. Leaf elongation, stomatal conductance and leaf water potential were again compared in treatments where roots were dried or severed. There was no difference from watered control plants if one pot was dried, in either mycorrhizal or non-mycorrhizal plants. If three pots or 3/4 of the root system were dried, all these parameters were reduced compared to control fully watered pots, and were thus hydraulically affected by root treatment, in both mycorrhizal or non-mycorrhizal plants. Non-mycorrhizal plants with two dried pots showed final leaf area and length reductions but not stomatal conductance, suggesting a non-hydraulic root-signalling response. Mycorrhizal symbiosis appeared to eliminate this root-signalled inhibition of leaf growth. However the difference in overall growth inhibition between half-dried mycorrhizal and non-mycorrhizal plants may have been related to differences in soil drying rate, when reductions in leaf extensions were compared to actual soil matric potential. The relative amount of the root system which experienced drying altered the response of the host plant, but the amelioration in this response by mycorrhizal association, which was seen in some of the work mentioned previously, was not seen here.

To some extent a quantitative response to drying of the hyphae and roots+hyphae portions of the root system, was achieved in this current study. The relative influence in water potential between each rhizobox section was compared. No conclusive evidence was seen in the relationship between gas exchange and relative degree of drying, between root+hyphal and hyphal portions.

If mycorrhizal hyphae could act as an extension of the host root system, it would be expected that there would be changes in the stomatal conductance with decreasing water availability, which were not due to a direct hydraulic response. Few studies report the length of extraradical hyphae because of the difficulty in obtaining accurate measurements (Green *et al.* 1994) It was suggested in this study that water loss from the hyphal section could be broadly related to hyphal length. If plant-fungal water relations were considered on a signalling basis, the length of hyphae extending into areas of soil beyond the roots, rather than the percentage mycorrhizal colonisation could be important in water relations. Mycorrhizal colonisation was

shown to be poorly related to external hyphal length. The strength of a signal might be proportional to the length of hyphae.

In the imposed treatments of hyphal severing or chemical control there were not enough replicate plants to show a pattern of plant gas exchange response to loss of external hyphae. No relationship between the relative gas exchange and hyphal length could be seen. In these analyses the hyphal length was categorised as above or below the group average. However there may be a critical quantity of hyphae necessary to give an altered response to drought stress in the plant. It is possible this could explain the differences in drought response of different host-fungal associations, as different species of mycorrhizal fungus are known to produce varying quantities of external mycelium (Abbott and Robson 1985).

Chemical nature of mycorrhizal root signalling

If external hyphae were responsible for signalling of drought conditions to the host plant shoot, then the chemical or range of chemicals involved in signalling should be examined. It has been shown that the likely chemical involved in plant root signalling is abscisic acid (ABA) (Zhang and Davies 1989,1990). The concentration of ABA in the roots was related to the soil water content. As soil dried, the ABA concentration in the xylem and epidermis of roots was increased (Neales *et al.* 1989, Zhang *et al.* 1987). This caused changes in the osmotic potential of xylem cells so that they showed increased turgor (Auge and Stodola 1990).

The alteration of mycorrhizal colonisation on plant water relations was also shown to be influenced by ABA concentration (Duan *et al.* 1996). A drought-avoiding plant, cowpea was inoculated with *G.intraradices*. At low water availability mycorrhizal plants maintained higher stomatal conductances, transpiration and shoot water potential. These higher foliar water status characteristics were associated with lower xylem sap concentrations of ABA, and ABA flux to leaves, in mycorrhizal plants. Stomatal conductance was closely correlated to ABA concentration. Mycorrhizal fungi probably increased the capacity of the root system to obtain water in drier soil, resulting in less stress in foliage and hence higher stomatal conductance at particular soil water content. This effect was also shown by Ebel *et al.* (1997), at

high water contents, when mycorrhizal plants had higher stomatal conductance, and lower xylem ABA. However the difference was not seen at low water contents due to stomatal closure. This was not related to plant water status because shoot water potential, xylem sap osmotic potential and shoot water content were similar for mycorrhizal and non-mycorrhizal plants, for all water contents. Stomatal sensitivity to ABA not affected by mycorrhizal symbiosis.

If the external mycelium was capable of signalling soil water content to the plant, it remains to be found whether this response also involved ABA or some other chemical. The presence of mycorrhizal fungi in the plant root causes several changes in the root chemistry. Larger amounts of amino acids have been found in mycorrhizal roots, during drought (Schellenbaum *et al.* 1998) and higher protein concentrations (Ruizlozano *et al.* 1996). Other detected compounds include alanine, aspararatine, glutamine and glycine (Subramanian and Charest 1995). Higher cytokinin were seen in leaves of mycorrhizal plants during drought (Goicoechea *et al.* 1995, 1996). AMF colonisation has been shown to increase the concentration of three isoflavonoid, stress metabolites (Morandi *et al.* 1984) and of indole-3-butyric acid (IBA), found in maize colonised by *Glomus intraradices* (LudwigMuller *et al.* 1997). Jasmonic acid is a plant growth regulator involved in stress signalling (Miersch 1997). It has been suggested that it may also influence mycorrhizal symbiotic relationships (Regvar and Gogala 1997). Regulatory substances from the host and from the fungus may act in balance with each other (Gogala 1997). The ratio of abscisic acid to cytokinin concentration in plant roots and shoots has been shown to increase under drought stress in non-mycorrhizal plants (Goicoehea et al. 1997). However in mycorrhizal plants this ratio was lowered under stress. Further work in this area might consider the following questions. What chemical changes occur in hyphae under dry conditions. What compounds are transported through hyphae which a plant might respond to? Cytoplasmic streaming in hyphae is a possible mechanism for transportation of the signalling compound. What compounds could be transported across the plant-fungal interface? It was not within the scope of this study to test these ideas, but provides the basis for biochemical explanations of the whole plant responses to drought, seen in mycorrhizal associations.

CHAPTER 6

Conclusions

Summary of results

Plant response to water deficit can be measured in a number of ways. These include the root and shoot morphology, rate of gas exchange, or tissue osmotic adjustment. The plant nutrient status can be implicated as the cause of these responses, or changes in plant hormones. The varying responses to drying in mycorrhizal plants previously found in the literature can be partly explained by differences in the parameters measured in different studies. The results found in this study are consistent with aspects of other work, such as increased biomass in mycorrhizal plants, some differing responses to decreasing soil water potential, and changes in micro-nutrient contents. In some cases such as shoot biomass, this difference in response is between mycorrhiza genera. In other cases such as root biomass and length, the variation is at the species level. Although *Gigaspora* species are not generally found in associations with *Populus*, this study showed that it increased the root length of the host plant. This feature could be beneficial in situations of drought stress or restricted rooting, such as soils of high clay content. Gas exchange in host plants was found to be reduced by mycorrhizal association during soil drying. This may have been a feedback response on stomatal conductance due to the increased leaf area of mycorrhizal plants, rather than an intrinsic reduction. No significant difference was found in the ability of mycorrhizal plants to continue to extract water from soil at lower water potentials than non-mycorrhizal plants. However there were indications that the association with mycorrhizal fungi was of greater significance to the host plant in periods of stress, when young or subjected to intense drying, rather than in periods of slight water deficit. There was some evidence that potential water uptake might be related to the quantity of external hyphae in the soil. There was no clear indication whether this was related to the metabolic activity of the hyphae. There may be a particular level at which mycorrhizal colonisation is useful in drought stress. This could be a soil water potential, a leaf water potential, an external hyphal length, or a time in a drought cycle. This was suggested by Savé *et al.*

(1994), where there was a threshold value in the soil water potential or conductivity through soil below which mycorrhiza colonisation is beneficial and above which it has detrimental effects on the plant water balance. There was slight evidence in my study that host gas exchange was influenced by the access of hyphae to wet volumes of soil. This was tentatively related to root to shoot signalling. It was suggested that external hyphae could be involved in signalling of drying soil conditions to the host, seen by a change in host gas exchange with and without access to these hyphae.

Achievement of objectives

Significant differences were found between three tree species in their water relations. However the pattern of water use was similar for all species, so that selection of a model species could be made on the basis of ease of handling of the plant material. The cuttings of *Populus* and the micropropagated *Prunus* were likely to be more uniform in growth than the seeds of *Acer*. In the majority of experiments, *Populus* was used because of the ease of production of plants. However the small size of *Prunus* proved useful in nutrient solution culture experimentation.

Differences in water use between species of trees could be adequately differentiated using a gravimetric method of measurement.

The control of the water potential of the root growth substrate was not achieved.

Culture of mycorrhizal plants in nutrient solution was shown to be successful with adequate aeration.

A plant chamber was designed and constructed to separate a portion of the external mycelium from a mycorrhizal plant root system. Prevention of root growth into this hyphal soil volume was successful.

Calibration of a fibreglass resistance sensor for measurement of soil water content required the inclusion of a temperature variable. Once this was included soil water content could be calculated and converted to soil water potential using a soil moisture release curve.

Conclusive evidence for differences in water uptake was not found between different species of mycorrhizae. There were differences in the carbon allocation

between genera and species of mycorrhiza. *Glomus* species showed a significant increase in shoot growth whereas *Gigaspora* species showed a decease. The only difference found in nutrient status between mycorrhizal and non-mycorrhizal plants was in potassium, which generally increased in mycorrhizal plants, and magnesium, which was lower in *Gi.rosea* colonised plants.

There was no evidence to suggest direct transport of water from the soil via external hyphae to the host plant in mycorrhizae.

There were indications of a whole plant response to changes in the external mycelium of the mycorrhizae. Leaf stomatal conductance appeared to increase in its range and maximum value when treatments were imposed on the external mycelium.

There was no conclusion to the mechanism of the plant response to its associated hyphae.

Methodology

There have been a very wide range of different microcosms used for mycorrhizal studies, particularly for studies on the external hyphae. There are two types of microcosms generally used. The first type involves allowing hyphae to grow through a mesh through which host plant roots cannot pass. The second type involves physically cutting off roots or hyphae from one section of a microcosm. Although interesting work has been done using the latter method, doubt remains as to whether the disturbance of the plant-fungal association, in terms of light, moisture, or physical damage to tissues, does not have an overwhelming influence on the experimental results. This is particularly true since changes in the environment are known to have an effect on mycorrhizal fungi. It must therefore be advantageous to use methods which maintain the association in an undisturbed environment. The aim of microcosms is to separate the influence of the hyphae from other indirect influences on the plant, not to alter the association itself with a change in environment. A number of studies have successfully employed mesh separation in mycorrhizal studies.

The length of hyphae able to pass the mesh is reduced from that found freely in pot studies. Clearly the mesh changes the proportion of hyphal length, relative to root length in a given volume of soil. This is inevitable in a microcosm which directs

tissue growth, that it will also limit it. However the limitation of hyphal tissue in the hyphal section of the rhizobox, was a major difficulty in this study. It also caused some further problems. In the use of a rhizobox for mycorrhizal studies, there is a trade-off between limitation of root growth and maximising the length of hyphae. The combination of host and fungus must influence this relationship. Ideally a plant with a highly plastic root system architecture would grow most successfully in a microcosm. Poplar root systems grew well in the rhizobox, fully utilising the soil volume. However the plants were also quickly subjected to extreme water deficit stress. This is suitable for water relations studies, but reduced the vigour of plants.

The plant material was seen to be highly variable in its root and shoot characteristics and in its response to imposed treatments. This may have been due to the tendency of the rooted cuttings to produce variable numbers of shoots and consequently biomass and leaf area. This feature made interpretation of experimental results more difficult. It indicated that much greater replication of treatments would be required to demonstrate adequately plant responses to mycorrhizal associations under drought stress.

Statistical analysis

The use of drying cycles and monitoring of changes in plant response with time influenced the method of statistical analysis. Treatment and time effects were confounded. Because of this analysis of variance could not be used successfully to determine treatment differences. It was however used successfully for discrete plant measurements such as nutrient concentration and biomass. Linear regression was a valuable tool in evaluating the effects of imposed treatments with time. It could be used to explain the variation in plant response with slight differences in their soil environment despite low numbers of replicates. Treatment effects could seen where variation between replicate plants hid these effects when analyses of variance were performed. Monitoring of treatment effects in the same plants over time may reduce the effects of between plant variation at any one point in time.

Further work

It could be said that there appeared to be a response in the plant transpiration to information from the hyphal section about water availability. However without greater numbers of replicate rhizoboxes, this relationship is at best tentative, and possibly a chance occurrence. It would therefore be of great interest to repeat the experiment and further test the idea of hyphal signalling to the host plant. The low numbers of plants limited the conclusions which could be reached from these rhizobox studies. There was some difficulty in controlling the soil water content of the hyphal section of the rhizobox. Because of this and the low number of replicates, comparisons could not be made where host roots external hyphae experienced very different soil water availabilities. The experiment should be repeated with treatments where the rhizobox sections were subjected to adequate water in both sections, drought in both sections, greater water availability in the root section and greater water availability in the hyphal section. This would allow a better evaluation of the relative importance of the external hyphae in water relations. In addition if the rhizobox sections were maintained at relatively constant soil water contents, the long-term effects of imposed treatments could be examined. The short-term effects could be seen in this study, but greater control of the water contents of each rhizobox section would be required to examine any long-term effects.

The relative proportion of internal and external hyphae and the alteration of internal structure of plants relative to external hyphal growth, could also be examined to explain the variation in drought response between different mycorrhiza.

These studies could be complimented with biochemical studies of plant growth regulators. AMF appear to alter the ratio of ABA, cytokinins and polyamines compared to non-mycorrhizal plants. Under water stress the ratio of these three regulators was related to nutrient levels in leaves, such as Ca, N, K and to leaf turgor (Sanchez-Diaz *et al.* 1997). Further studies in mycorrhizal plant water relations should consider the alteration in plant growth, particularly the relationship between ABA, cytokinins and polyamines.

Appendix 1

Soil Characteristics

The following analyses were carried out in the Department of Plant and Soil Science, Aberdeen University, from Povey (1994) PhD Thesis

Craibstone soil

Series	Countesswells
pH in water	6.46
Cation Exchange Capacity	7.4 cmol kg^{-1}
Texture	
Sand	73.85%
Silt	20.03%
Clay	6.12%
Texture	Loamy sand
Organic Matter	4.25%
Organic Carbon	3.79%
Organic Nitrogen	272.7 mg $100g^{-1}$ soil
Available Nitrogen	6.8 mg $100g^{-1}$ soil
Biomass Carbon (direct extraction)	101.8 mg C $100g^{-1}$ soil

The following analyses were carried out at in the Environmental Sciences Department, SAC, Ayr

Aldroughty soil

pH (in water)	6.0
Cation Exchange Capacity	2.9 cmol kg^{-1}
Texture	
Sand	85.4%
Silt	11.3%
Clay	3.3%
Texture	Loamy sand
Organic Matter	2.22%
Organic Carbon	1.28%
Organic Nitrogen	120 mg $100g^{-1}$ soil
Extractable Phosphorus (acetic acid)	469 mg l^{-1}

Appendix 2

Hoaglands Nutrient Solution

	g l^{-1}
KNO_3	0.51
$Ca(NO_3)_2$	0.82
$MgSO4.7H_2O$	0.49
KH_2PO4	0.136
Ferric tartrate	1 ml l^{-1} of 0.5% solution
Hoaglands A-Z Micronutrients	1 ml l^{-1}

Hoaglands A-Z Micronutrients

	g l^{-1}
H_3BO_3	2.86
$MnCl_2.4H2O$	1.81
$ZnSO_4.7H2O$	0.22
$CuSO_4.5H2O$	0.08
$H_2MoO_4.2H2O$	0.025

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